White Paper on Potential Developmental Effects of Atrazine on Amphibians

In Support of an
Interim Reregistration Eligibility Decision
on Atrazine

Submitted to the FIFRA Scientific Advisory Panel
for Review and Comment
June 17 - 20, 2003

Office of Prevention, Pesticides and Toxic Substances
Office of Pesticide Programs
Environmental Fate and Effects Division
Washington, D. C.

May 29, 2003

ACKNOWLEDGMENTS

Authors:

Thomas Steeger, Office of Prevention, Pesticides, and Toxic Substances, Office of Pesticide Programs, Environmental Fate and Effects Division and Joseph Tietge, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division were responsible for preparation of the white paper.

Reviewers:

The authors acknowledge the input of a number of reviewers. Reviewers included: Les Touart and Joseph Merenda from the Office of Science Coordination and Policy; William Jordan from the Immediate Office of Pesticide Programs, Kimberly Nesci (Special Review and Reregistration Division); Arthur-Jean Williams (Field and External Affairs Division); and Mary Frankenberry, Stephanie Irene, Karen McCormack, Edward Odenkirchen, Ingrid Sunzenauer, and Douglas Urban (Environmental Fate and Effects Division) from the Office of Pesticide Programs. Additionally, Gary Ankley, Sigmund Degitz and Patricia Schmieder of the Mid-Continent Ecology Division, Office of Research and Development and Nancy Beck of the Office of Information and Regulatory Affairs, Office of Management and Budget provided helpful reviews.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

BACKGROUND

In response to an amended consent decree entered to conclude litigation between EPA and the Natural Resources Defense Council (NRDC), the Agency released on, January 31, 2003, an atrazine Interim Reregistration Eligibility Decision (IRED) that was based, in part, on an assessment of the pesticide's ecological risk. However, several studies concerning the potential effects of atrazine on amphibian development were published in a time frame that prevented the Agency from completing a rigorous evaluation of these data for inclusion in the January 31, 2003 IRED.

Thus, the decree stipulated that "EPA shall sign, on or before October 31, 2003, a revised IRED for atrazine that addresses . . . data, received by EPA prior to February 28, 2003, relating to the potential effects of atrazine on amphibian species." The Consent Decree also stipulated that "[a]t least three months prior to signing this revised IRED, EPA shall develop a paper and submit it to the FIFRA Scientific Advisory Panel (SAP) for review and comment." The paper is required to address "the significance of the amphibian risk data" and "whether there is a need for additional data to characterize more fully atrazine's potential risks to amphibian species, and, if so, what data should be developed."

In accordance with the consent decree, EPA has developed this white paper as the basis for an SAP meeting to be held on June 17 - 20, 2003. The white paper provides an overview of relevant studies published in the scientific literature and studies submitted by the registrant through February 28, 2003. This white paper assesses:

- the strengths and limitations of the available studies to evaluate the extent to which atrazine may elicit developmental effects in amphibians;
- the extent of concordance for the entire body of information derived from these laboratory and field studies to assess the plausibility that atrazine can cause developmental effects; and if so,
- the nature and strength of associated dose-response relationships.

The paper also outlines a conceptual model for potential future studies that could provide information to resolve inconsistencies and address gaps in the existing knowledge base that currently preclude establishing a definitive characterization of atrazine's effects on amphibian development.

OVERVIEW OF LABORATORY AND FIELD STUDIES

In developing this white paper, EPA reviewed a total of 17 studies (**Table A**) that were published in the scientific literature or submitted by the registrant. Seven were laboratory-based investigations, and ten were field studies. Of the twelve registrant-sponsored studies, seven were considered preliminary or in progress by the registrant. All studies were individually evaluated with regard to the following parameters: experimental design, protocols and data quality assurance, strength of cause-effect and/or dose-response relationships, mechanistic plausibility, and ecological relevancy of measured endpoints.

Laboratory Studies

The laboratory studies reviewed for this paper addressed a variety of endpoints: time to metamorphosis, growth (body length and weight), gonadal abnormalities, sex ratios, laryngeal dilator muscle area, plasma steroid concentration, and brain/gonad aromatase activity. In evaluating the effects of atrazine on amphibian development, researchers tested a variety of frog species, including the African clawed frog (*Xenopus laevis*), the leopard frog (*Rana pipiens*), and the green frog (*R. clamitans*). Not all species were used in every study. All of the laboratory studies used static renewal exposures. Within individual studies the number of atrazine exposures ranged from only one concentration to up to six different concentrations. In some cases, the nominal atrazine concentrations were confirmed analytically.

The Agency's evaluation of the relevant studies in the open literature and registrant-submitted laboratory investigations concludes that none of the laboratory studies fully accounted for environmental and animal husbandry factors capable of influencing endpoints which the studies

were attempting to measure. For example, in several studies excessive mortality and/or delayed development in untreated organisms or a failure of organisms to respond to a positive control (*e.g.*, estradiol) confounded the interpretation of study results. In addition, in all the studies, loading rates (*i.e.*, tadpole biomass per liter of water) were typically too high for the solution changes associated with static renewal atrazine exposures. Resulting poor water quality (*e.g.*, low dissolved oxygen, high ammonia) could have created environmental conditions unfavorable to optimum survival, growth and development. In other instances, the condition of the organisms may have been compromised by poor quality feed.

After reviewing the entire body of information available from the laboratory investigations, EPA has concluded that the scientific evidence does not support many of the conclusions reached by the various study authors. Some of the major limitations of these laboratory studies, that make it difficult to draw conclusions with confidence about the effects of atrazine on amphibian species, include the following:

- For studies conducted at atrazine concentrations in the range of 0.1 to 25 ug/L, the interpretation of dose-response relationships for measured endpoints (*e.g.*, gonadal abnormalities, aromatase activity, plasma steroid concentrations and laryngeal dilator muscle diameter) was problematic. Analytical measurements of atrazine were incomplete, or atrazine was detected in the dilution water for the control organisms at concentrations comparable to low concentration treatments.
- Gonadal abnormalities (ovotestes and discontinuous gonads) and laryngeal dilator muscle diameter effects, reported at atrazine concentrations in the range of 0.1 to 200 ug/L, have not been reproduced to date. The extensive variability in the study design protocols has made it difficult to determine if this lack of reproducibility for demasculinizing effects (decreased laryngeal dilator muscle area), and reports of an inverted dose-response relationship for other gonadal developmental endpoints, are valid results or artifacts of the design and quality of the investigations.
- The potential gonadal developmental effects of atrazine, as well as an explanation for the non-monotonic dose-response curve reported by some investigators, has been proposed to result from induction of aromatase activity. This increased enzyme activity would in turn

lead to elevated estrogen levels and ultimately the observed effects, *i.e.*, ovotestes and reduced secondary sex characteristics, in males. Aromatase induction by atrazine, though, has not been demonstrated in any anuran in controlled laboratory investigations.

Field Studies

The field studies, which included a microcosm experiment, evaluated growth (body weight and length), gonadal abnormalities, sex ratios, plasma steroid concentration and brain/blood aromatase activity in *X. laevis*, *R. clamitans*, cricket frogs *(Acris crepitans*), bullfrogs (*R. catesbiena*), and cane toads (*Bufo marinus*) in Florida, Indiana, Iowa, Michigan, Nebraska, Utah, and Wyoming, USA and South Africa.

The Agency also concludes that the currently available field studies are of limited value due to the high variability in environmental conditions (*e.g.*, photoperiod, temperature, water quality) under which field-collected organisms lived, uncertainty as to amphibian developmental status and condition at the initiation of the studies, and an inability to relate the co-occurrence of atrazine with key developmental windows for the organisms under investigation. Consequently, EPA cannot determine whether the failure of several studies to show any relationship between measured or predicted aqueous atrazine concentrations and developmental effects reflects the absence of a causal relationship or the limitations of the study methodologies. In addition, the actual or possible co-occurrence of additional chemical and/or non-chemical stressors confound attempts to attribute any observed responses to atrazine exposure.

Conclusions from Laboratory and Field Studies

Overall, the weight-of-evidence based on currently available studies does not show that atrazine produces consistent, reproducible effects across the range of exposure concentrations and amphibian species tested. The current body of knowledge has deficiencies and uncertainties that limit its usefulness in interpreting potential atrazine effects. Specifically, the demasculinizing (decreased laryngeal dilator muscle area) effects were not replicated in multiple laboratories.

Additionally, the feminizing effects (intersex/hermaphroditism/ovotestes) of atrazine were observed in three laboratory studies whose experimental designs could not be easily reconciled and that reported significant effects at different concentrations: one at 25 ug/L atrazine and the other two at 0.1 ug/L. While the feminizing effects observed in these different studies were consistent qualitatively, there was no consistency across the studies in the reported dose-response relationships. That inconsistency, together with the limitations in methodology in each study, does not allow a reliable determination of causality or the nature of any dose-response relationship. Although the Florida cane toads monitored in the field exhibited both demasculinizing effects (genetic males with female coloration) and feminizing effects (oogenesis in male Bidder's organ), there were insufficient data to conclusively link atrazine exposure to the phenomena. Thus, the available data do not establish a concordance of information to indicate that atrazine will or will not cause adverse developmental effects in amphibians.

Conceptual Model for Future Studies

If the Agency's risk management decision requires a greater degree of certainty in the ecological risk assessment for atrazine than possible from currently available data, then additional information would be necessary to evaluate the potential causal relationships between atrazine exposure and gonadal development in amphibians. If a causal relationship is indicated, then further testing may be needed to establish the nature of the dose-response relationship, the ecological relevancy of the effects, the plausibility of an underlying mechanism(s), and the degree of interspecies variability for any effects.

Laboratory-based studies, planned and executed to address critical design and protocol issues, can provide data to adequately assess whether atrazine exposure results in gonadal developmental effects in frogs, and if so, the strength of a cause-effect relationship(s) and the nature of the underlying dose-response relationship(s). Because field investigations may introduce considerable variability and make it difficult to establish cause-effect relationships, EPA recommends that more controlled laboratory studies be conducted before field studies are undertaken.

EPA proposes that the studies be conducted in a tiered approach to ensure that the potential effects of atrazine are evaluated in a systematic and efficient manner, thus minimizing the level of effort and resources required, while maximizing reductions in the extant uncertainties. The first objective in this approach would be to determine the effects of atrazine on gonadal developmental endpoints at the organism level (ovotestes, sex ratios), using high quality aquatic toxicology methods with *X. laevis* and, to a lesser degree, North American *Ranids*. These studies would adhere to American Society for Testing Materials (ASTM) recommended standards for loading rates, husbandry and water quality parameters. The studies would address inconsistencies in previous research regarding the causal relationship between atrazine exposure and developmental effects and would provide sufficiently robust data to determine the nature of any dose-response relationship.

If the results from the initial studies are negative, then there would be no rationale to conduct further investigation. However, if the results are positive, then EPA proposes that additional studies would be designed to investigate plausible mechanisms involved in the etiology of the response. Establishing a mechanistic rationale is a critical basis for inter-species extrapolation. Mechanistic understanding would permit the development of bioindicators that can be applied to future field studies, if deemed necessary, and it would reduce the uncertainties associated with developing a causal relationship between effects on measurement endpoints and atrazine exposure. Assuming that the Panel reaches conclusions consistent with those proposed in the current review and evaluation of the existing data, EPA may request a consultation with the SAP to review in greater detail study designs and protocols for any higher tiered studies.

Table A. Summary of studies evaluated on the effects of atrazine on amphibian gonadal development and sexual differentiation.

Study First Author	EPA Study Number or Open Literature Citation	Species	Study Type
Hayes *et al.* 2002*a*	*Proceedings of the National Academy of Sciences*, 99:5476-5480	*Xenopus laevis*	laboratory
Goleman *et al.* 2003	MRID No. 458677-07	*Xenopus laevis*	laboratory
Tavera-Mendoza *et al.* 2001*a*	*Environmental Toxicology and Chemistry*, 21: 527-531	*Xenopus laevis*	laboratory
Tavera-Mendoza *et al.* 2001*b*	*Environmental Toxicology and Chemistry*, 21: 1264-1267	*Xenopus laevis*	laboratory
Hecker *et al.* 2003	MRID No. 458677-04	*Xenopus laevis*	laboratory
Hecker *et al.* 2003	MRID No. 458677-03	*Rana clamitans*	laboratory
Villeneuve *et al.* 2003	MRID No. 458677-08	*Xenopus laevis*	laboratory
Hayes *et al.* 2002*b*	http://dx.doi.org/ *[Online 23 October 2002] Environmental Health Perspectives*, 111(4): 568-575	*Rana pipiens*	laboratory and field
Du Preez *et al.* 2003	MRID No. 458677-11	*Xenopus laevis*	microcosm
Giesy *et al.* 2003	MRID No. 458675-01	*Xenopus laevis*	field
Smith *et al.* 2003	MRID No. 458677-01	*Xenopus laevis*	field
Smith *et al.* 2003	MRID No. 458677-09	*Xenopus laevis*	field
Smith *et al.* 2003	MRID No. 458677-10	*Xenopus laevis*	field
Crabtree *et al.* 2003	MRID No. 458677-05	*Rana catesbiena*	field
Jones *et al.* 2003	MRID No. 458677-02	*Rana clamitans*	field
Sepulveda *et al.* 2003	MRID No. 458677-06	*Bufo maninus* *Bufo terrestris*	field
Reeder *et al.* 1998	*Environmental Health Perspectives*, 106: 261 - 266	*Acris crepitans*	field

CHAPTER 1

INTRODUCTION

Regulatory Background

In April 2002, the Office of Pesticide Programs (OPP) of the U. S. Environmental Protection Agency (EPA) issued a document that characterized the environmental fate and ecological effects of atrazine in support of the interim reregistration eligibility decision (IRED) on atrazine (IRED Science Chapter 2002). On April 16, 2002, EPA held a technical briefing where Agency officials discussed human health and ecological risk assessments for atrazine. At the same time as the public briefing, newly generated information regarding the potential effects of atrazine on amphibian development was published in the open literature (Hayes *et al*. 2002*a*), and concerns were raised that EPA had not sufficiently accounted for the potential effects of atrazine on amphibian development and possible effects on human health.

In an amended consent decree (Consent Decree 2002) between EPA and the Natural Resources Defense Council (NRDC), EPA was required to issue, by January 31, 2003, an IRED on atrazine; the consent decree acknowledged that the IRED would not take into account recent literature on the effects of atrazine on amphibians. The decree further stipulated that EPA sign, by October 31, 2003, a revised IRED that incorporated recommendations from a FIFRA Scientific Advisory Panel (SAP) regarding studies on the potential effects of atrazine on amphibians. The decree indicated that EPA would review data received by the Agency prior to February 28, 2003, and that the SAP would review a paper developed by EPA which described and evaluated these studies. In accordance with this consent decree, EPA has developed a white paper that critically evaluates currently available data, discusses the nature of remaining uncertainties in evaluating the potential effects of atrazine on amphibian development, and outlines the nature of future studies that will address these uncertainties.

Summary of Pesticide Effects Characterization

Although EPA routinely requires testing in multiple species (40 CFR 158), pesticide toxicity data are not likely to be available for all potentially exposed non-target organisms. In the majority of cases, the risk assessment process relies on a suite of toxicity studies performed on a limited number of surrogate organisms. For example, mallard duck (*Anas platyrhynchos*) and bobwhite quail (*Colinus virginianus*) are common surrogates for birds, while rat and mouse strains, typically used in studies to support human health risk assessments, serve as surrogates for mammalian wildlife. With regard to aquatic life associated with freshwater ecosystems, typical surrogate species include the water flea (*Daphnia magna*), bluegill sunfish (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*), and fathead minnow (*Pimephales promelas*).

Within these broad taxonomic groups, one acute and one chronic endpoint (usually mortality and frank measures of growth and reproduction, respectively) are selected from the available test data. Data from the most sensitive species tested within that taxonomic group are selected. If additional toxicity data for more species of organisms in a particular group are available, the selection need not be limited to the species mentioned previously, but may be expanded to include additional data that meet the Agency's data quality requirements. Regardless of the extent of data available beyond the required set of toxicity studies, the risk assessment typically relies on the selection of endpoints from the most sensitive species tested in acceptable studies.

While the above mentioned surrogates and toxicity endpoints are routinely used in Agency risk assessments, they do not represent a limitation on the types of toxic endpoints that may be considered in the risk assessment. Through the evaluation of available effects data, the EPA risk assessment team may encounter other effects information that provides insight on endpoints and organisms not routinely considered. Professional judgment is used by the risk assessment team to determine whether and how available data on other toxicological endpoints are included in the risk assessment. This evaluation includes reference to data quality objectives for specific types of studies, the degree to which adequate documentation is available to evaluate the technical merit of the data, and whether the data are applicable to the assessment endpoints established for the risk assessment. In deciding whether data are applicable to assessment endpoints, the risk assessment

team uses professional judgment and available lines-of-evidence to determine if the toxicological endpoints can be linked to assessment endpoints in a reasonable and plausible manner.

Consistent with the previous discussion on endpoint selection, the selection of amphibian species as the ecological entity for a risk assessment endpoint may be appropriate based on a variety of considerations, such as a pesticide's mode of action, its use patterns and the species habitat requirements. Because amphibians undergo profound changes during metamorphosis and occupy a variety of ecological niches during their life-span, specific species, or groups of species, may be relevant entities for formulating risk assessment endpoints for certain pesticides. Amphibians exhibit a life cycle in which conditions must be favorable for their survival in aquatic ecosystems where they breed and larvae develop, and in terrestrial habitats, where many adult amphibians reside. Living first as gilled- and skin-breathing aquatic larvae and later as completely or partly land-dwelling lung- and skin-breathing adults, these animals rely on a wide range of mechanisms for interfacing with their environments. Unlike many organisms that have either keritinized and/or scaled outer surfaces, the amphibian skin is thin and is used as a respiratory membrane for gas exchange and water absorption. During the larval stage, many amphibians rely on filter feeding although, as a group, they are considered the primary vertebrate predator for invertebrates in many freshwater and moist terrestrial environments.

Background on Atrazine

Atrazine is a selective, pre- and post-emergence herbicide used on a variety of terrestrial food crops, non-food crops, forests, residential turf, golf course turf, recreational areas, rights-of-way, and rangeland. Although used to control broadleaf and many other weeds on a range of agricultural and nonagricultural sites, the herbicide's largest use is on corn, sorghum and sugarcane. Atrazine's primary mode of action in plants is through inhibition of photosynthesis by disruption of the Photosystem II pathway. Atrazine is characterized as a persistent, mobile compound that may be transported to surface water via runoff, spray drift, and atmospheric deposition (IRED 2003). Because of the chemical's resistance to abiotic routes of degradation, and its moderate susceptibility to biotic degradation, it is likely to persist in water. These characteristics, combined with the herbicide's level of use, have contributed to its widespread

detections in surface waters throughout the United States and its associated exposure potential for aquatic organisms.

Although atrazine exhibits varying degrees of acute and chronic toxicity to animals, its major identified impact on aquatic organisms appears to be an indirect effect resulting from a decrease in primary productivity in associated ecosystems (*i.e.*, decreased plant biomass). In terms of direct effects, atrazine is moderately toxic to fish (LC_{50} = 5.3 mg/L) and highly toxic to aquatic invertebrates (LC_{50} = 0.72 mg/L) on an acute exposure basis. Although screening-level assessments did not show an acute risk to aquatic animals, the level of concern for chronic risk to fish and invertebrates was exceeded for certain uses of atrazine. With respect to terrestrial organisms, atrazine was found to be slightly toxic to birds (LD_{50} = 940 mg/kg) and mammals (LD_{50} =1,869 mg/kg) on an acute exposure basis (IRED Science Chapter 2002). Chronic sublethal effects in mammals included effects on the hypothalamic-pituitary axis in rats (HED Science Chapter 2002), where prolonged atrazine treatment attenuated pre-ovulatory lutenizing hormone surge thereby inhibiting ovulation and potentially delaying the onset of puberty for both males and females.

In April 2002, Hayes *et al.* (2002*a*) reported in the *Proceedings of the National Academy of Sciences* that exposure to atrazine concentrations as low as 0.1 µg/L resulted in demasculinization (reduced laryngeal muscle growth) and feminization (testicular oogenesis/hermaphroditism) of African clawed frogs (*Xenopus laevis*) in the laboratory. Studies previous to those of Hayes *et al.* (2002*a*) reported the incidence of hermaphroditism in cricket frogs (*Acris crepitans*) correlated with atrazine exposure (Reeder *et al.* 1998). Other studies showed that exposure of *Xenopus laevis* to a single concentration of atrazine (21 µg/L) increased the frequency of secondary oogonia and atresia of both primary and secondary oogonia in ovaries (Tavera-Mendoza *et al.* 2001*a*) and reduced testicular volume, spermatogonia, and nurse cells in testes (Tavera-Mendoza *et al.* 2001*b*).

Sanderson *et al*. (2002) reported that atrazine up-regulated the cytochrome P450 CYP-19 gene in human cancer cell lines and increased synthesis of aromatase. According to the authors, increased aromatase levels could, in turn, increase the baseline conversion of testosterone to estrogen. Additional studies published by Hayes *et al.* (2002*b, c)* in *Environmental Health Perspectives* and *Nature* reported that similar effects on gonadal development were achieved using the non-native *X. laevis* and the native leopard frog (*Rana pipiens*). Finally, field studies

reported that leopard frogs collected in atrazine-exposed sites exhibited testicular oogenesis and hermaphroditism at rates as high as 92% (Hayes *et al.* 2002*b*, *c*). In response to this published literature, the registrant (Syngenta Crop Protection, Inc.) sponsored a series of studies to further examine the potential for atrazine to affect development in both native and non-native frog species. The primary focus of these registrant-sponsored studies was to examine gonadal development, laryngeal muscle growth, blood testosterone and estrogen levels, and gonadal and/or brain aromatase activity in frogs exposed to atrazine.

The intent of this white paper is to critically review the available open literature and registrant-sponsored studies that examine the effects of atrazine on amphibians and to evaluate whether there is sufficient information to conclude that atrazine exposure does or does not result in toxic effects on amphibian development. In this white paper, Chapter 1 provides an overview of the regulatory history of atrazine, the Agency's process for evaluating information used in characterizing potential risks associated with the use of pesticides, and the exposure/effects characterization of atrazine. In Chapter 2, actual study reviews are then grouped into laboratory and field investigations; study methods and results are presented followed by a discussion of specific issues associated with each study. Following these reviews, Chapter 3 discusses common strengths and limitations found in the laboratory and field studies. Based on the study reviews and their associated strengths and limitations, major uncertainties are identified in Chapter 4 that could limit the usefulness of the studies in charactering the potential developmental effects of atrazine on amphibians for the purposes of risk assessment. Chapter 5 provides recommendations on how various uncertainties could be addressed, and Chapter 6 presents the conclusions of the paper. Finally in Chapter 7, EPA poses a series of questions that require input from the SAP on the Agency's study evaluations and recommendations to reduce uncertainties related to establishing a causal relationship between atrazine exposure and effects on amphibian development.

CHAPTER 2

STUDY REVIEWS

In response to studies published in the open literature regarding the potential effects of atrazine on amphibian development, Syngenta sponsored a series of studies conducted by the Atrazine Endocrine Ecological Risk Assessment Panel administered by Ecorisk, Inc. of Ferndale, WA. According to the registrant, the studies were initiated to investigate potential endocrine effects of atrazine in wildlife species, including amphibians. The studies were submitted by February 28, 2003 and were provided specifically for consideration at the FIFRA Scientific Advisory Panel's meeting in June 2003.

In the following sections, summary reviews, grouped into laboratory and field studies, are presented for each individual study. Each study was individually evaluated with regard to experimental design, protocols and data quality assurance, strength of cause-effect and/or dose-response relationships, mechanistic plausibility, and ecological relevancy of measured endpoints. Throughout the reviews, the terms intersex, hermaphroditism, and ovotestes were used interchangeably and refer to situations where ovarian and testicular tissue were observed in the same animal/gonad.

Consistent with EPA's process for evaluating scientific studies, the Agency completed data evaluation records (DERs) for each of the Syngenta-sponsored studies and conducted a critical review of the pertinent studies reported in the open literature. In the DERs for the registrant-sponsored studies, the Agency reviewers outlined the study methodologies, re-analyzed raw data, and documented any uncertainties or differences in conclusions from those of the study authors. It is important to note that the review of these registrant-sponsored studies was more detailed than for the studies obtained from open literature. For these latter studies, the reviews were less detailed because EPA did not have access through the study authors to the full range of raw data and quality control information required for registrant-submitted studies.

Laboratory Studies

One of the advantages of conducting laboratory studies is that they allow researchers to control a range of conditions that could potentially impact the outcome of a study. Environmental factors, water quality, loading rate, chemical exposure, study animals, animal husbandry and health can all be manipulated more easily to identify actual treatment effects in laboratory studies. Laboratory studies also allow greater flexibility in study design to account for known sources of variability. For example, sample size and replication can be manipulated to reduce confidence intervals around treatment means. Another advantage of laboratory studies is that researchers can use positive controls to gauge the responsiveness of the test organisms to treatment effects. Finally, laboratory studies facilitate sample processing since analyses can be conducted in close proximity to where samples are collected.

This chapter includes a review of seven laboratory studies, three taken from open literature and the remainder sponsored by the registrant. A summary of important attributes of these studies is presented in **Table 1**. Although **Table 1** depicts nine studies, two of the studies were field studies that had laboratory components.

Table 1. Summary of basic conditions and findings of various laboratory or controlled exposure studies on the effects of atrazine on amphibian development.

Study First Author	Study Number or Citation	Study Type	Species	Length of Exposure	Developmental Stage	Nominal Exposure Concentrations (µg/L)	Primary Endpoints	Significant Atrazine Effects as Determined by Authors and Associated Atrazine Concentration	
								Effects	(µg/L)[3]
Hayes	PNAS, 99:5476-5480	static renewal	*X. laevis*	nr	initial: NF46 final: NF66	0.01, 0.1, 1.0, 10, 25 0.1, 0.4, 0.8, 1.0, 25, 200	testicular morphology laryngeal muscles	↑ ovotestes ↓ laryngeal dilator size	0.10 1.0
				46 d	initial: adult final: adult	25	plasma testosterone	↓ plasma testosterone	25.0
Goleman	EPA MRID: 458677-07	static renewal	*X. laevis*	78 d	initial: 48 h ph final: NF66	1.0, 10, 25	testicular morphology ovarian morphology laryngeal muscles	↑ ovotestes	25.0
Du Preez [4]	EPA MRID: 458677-11	static microcosm	*X. laevis*	133 d	initial: 120 h pf final: juveniles	1.0, 10, 25	testicular morphology ovarian morphology	none	na
Tavera-Mendoza	ET&C, 21: 527-531	static	*X. laevis*	48 h	initial: NF56 final: NF56	21	testicular development	↓ testes size ↓ spermatogonial nests ↓ nurse cells	21.0 21.0 21.0
Tavera-Mendoza	ET&C, 21: 1264-1267	static	*X. laevis*	48 h	initial: NF56 final: NF56	21	ovarian development	↓ primary oogonia ↑ secondary oogonia ↑ atresia	21.0 21.0 21.0

Study First Author	Study Number or Citation	Study Type	Species	Length of Exposure	Developmental Stage	Nominal Exposure Concentrations (µg/L)	Primary Endpoints	Significant Atrazine Effects as Determined by Authors and Associated Atrazine Concentration	
								Effects	(µg/L)[3]
Hecker	EPA MRID: 458677-04	static renewal	*X. laevis*	185 d	initial: 72 h ph final: juvenile	0.1, 1.0, 10, 25	testicular morphology ovarian morphology gonad aromatase brain aromatase plasma estradiol plasma testosterone laryngeal muscles	none	na
Villeneuve	EPA MRID: 458677-08	static renewal	*X. laevis*	26, 43, 47 d	initial: adult final: adult	25	gonad aromatase brain aromatase	none	na
Hayes[1]	EHP, 111: 568-575	static renewal	*R. pipiens*	nr	initial: 48 h ph final: juvenile	0.1, 25	testicular morphology	↑ ovotestes ↑ dysgenesis	0.10 0.10
Hecker	EPA MRID: 458677-03	static renewal	*R. clamitans*	273 d	initial: NF46[2] final: NF66[2]	10, 25	testicular morphology ovarian morphology	none	na

[1] Also reviewed as field study (information presented in table represents laboratory data only); [2]NF stage used for convenience, [3] Nominal concentration,

[4]Reviewed as field study Abbreviations: nr: not reported; na: not applicable; ph: post hatch; pf: post fertilization; NF: Nieuwkoop and Faber Stage;

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk. 2002a. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. ***Proceedings of the National Academy of Sciences*** **99 (8): 5476 - 5480.**

The objective of this study was to test whether atrazine interfered with metamorphosis and sex differentiation at environmentally relevant, low doses via endocrine-disrupting mechanisms. Using three replicates with 30 tadpoles per replicate, *Xenopus laevis* were exposed under static renewal conditions (complete exposure water change every 72 hrs) to nominal atrazine concentrations ranging from 0.01 to 200 µg/L from 96-hr post-hatch through complete tail resorption (Nieuwkoop-Faber (NF) Stage 66[1]) (Nieuwkoop and Faber 1994). Animals were maintained in plastic containers typically used to house laboratory mice (personal communication: Tyrone Hayes, University of California at Berkeley, 2002); containers were filled with 4 L of exposure solution. At the end of the exposure period, animal growth (length and weight), time to metamorphosis, gonadal abnormalities and size (cross-sectional diameter) of the larynx dilator muscle were recorded.

Exposure to atrazine concentrations ≥ 0.1 µg/L resulted in gonadal abnormalities in 16 - 20% of the animals. The actual incidence of gonadal abnormalities at each exposure level was not reported. For this reason, the quantitative relationship (dose-response) between atrazine concentration and gonadal effects is uncertain. Abnormalities included multiple gonads or hermaphrodites (multiple testes and ovaries in the same animal); these abnormalities were not observed in controls. Although males typically exhibited larger laryngeal muscle diameters than females, atrazine exposure ≥ 1 µg/L significantly decreased (G test[2], $p < 0.05$) the proportion of males that were at or above the mean control for males. This finding suggested a threshold effect

[1]Nieuwkoop and Faber (1994) define Stage 66 of *X. laevis* development as the point at which the tail is only represented by small dorsal swelling of loose connective tissue, covered with degererating larval skin; the tail [is] only a very small triangle, no longer visible from ventral side.

[2]The G-test (or log-likelihood ratio test) of goodness-of-fit, may be used to test the fit of data in treatments or classes against proportions expected from control group(s) or from historical reference data. The null hypothesis is that the observed frequencies fit a particular ratio (50:50, or 3:1, etc.); a significant result indicates that the observed frequencies are different from the expected ratio.

at ≥ 1 µg/L in which 80% of the exposed males had below average laryngeal muscle diameters. In this study, Hayes *et al.* (2002*a*) hypothesized that the co-occurrence of oocytes and testicular tissue (hermaphroditism) and the decreased male larynx muscle size (demasculinization) were consistent with increased endogenous estrogen concentrations. The authors proposed that one possible mechanism for increased estrogens would be through increased aromatase activity. In support of this, Hayes *et al.* (2002*a*) reported that an average adult male *Xenopus* exposed to atrazine at 25 µg/L had significantly reduced plasma testosterone.

This study states that the results have been repeatedly verified, but additional data have not yet been provided in the open literature or submitted to EPA[3] The lack of a dose-response relative to the phenomenon of hermaphroditism raises problems for interpreting cause-effect relationships. Although there appears to be a dose-dependent reduction in laryngeal muscle area relative to atrazine concentrations, the reliance on the proportion of animals falling below average is an indirect measure of the effect. A direct comparison of measured laryngeal muscle area between controls and exposed animals would further clarify the magnitude and extent of any developmental effects. Information on how diminished dilator muscle area or the gonadal abnormalities might relate to the reproductive success, growth or survival of the affected species in the environment would also provide further insights on the ecological relevancy of effects.

This study was useful in identifying a potential hazard to amphibians and presented information on measurement endpoints, such as gonadal deformities and laryngeal muscle diameter. The study, however, did not show a clear dose-response that demonstrates a causal relationship between atrazine exposure and amphibian developmental effects.

[3]The Agency did not receive data on these additional studies and was not able to verify these conclusions.

Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001*a*. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the testes. *Environmental Toxicology and Chemistry* 21 (3): 527 - 531.

In an effort to examine the effects of atrazine on gonadal differentiation and reproductive impairments, male *X. laevis* (NF Stage 56) were exposed to mean-measured concentrations of atrazine at 18 µg/L (nominal: 21 µg/L) under static conditions for 48 hrs. Animals (16 larvae per replicate with 2 replicates per treatment) were fasted during the 48-hour test. Total testicular volume decreased significantly ($p = 0.004$) from 0.026 ± 0.003 mm^3 in controls to 0.01 ± 0.001 mm^3 in atrazine-treated tadpoles, representing a 57% decrease after 48 hrs of exposure. The number of spermatogonial cell nests decreased significantly ($p < 0.001$) from a mean of 242.4 ± 35.7 in controls to 72.9 ± 21.8 in atrazine-exposed tadpoles, representing a 70% reduction. The number of nursing cells declined significantly ($p < 0.001$) from a mean of 9.62 ± 0.17 in controls to 2.35 ± 0.36 in atrazine treated tadpoles. Testicular resorption was observed in 70% of the male tadpoles exposed to atrazine relative to controls; failure of full development of the testis (aplasia) was observed in 10% of the testes examined. Histological examination of the pituitary suggested that tissues were actively secreting hormones based on the absence of chromophores.

This study provides useful information on hazard identification and measurement endpoints, such as gonadal abnormalities. However, the study does not provide sufficient information to establish a dose-response relationship because only one concentration of atrazine was tested. Other information which is needed to more fully interpret the significance of this study include: documentation of the measurement endpoints, elaboration on the ecological relevancy of the measurement endpoints, and clarification of the number of exposed animals.

Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001*b*. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. *Environmental Toxicology and Chemistry* 21 (6): 1264 - 1267.

To examine the effects of atrazine on gonadal differentiation during larval tadpole development of female *X. laevis*, NF Stage 56 tadpoles were exposed to mean-measured concentrations of atrazine at 18 µg/L (nominal: 21 µg/L) under static conditions for 48 hrs. Animals (16 larvae per replicate with 2 replicates per treatment) were fasted during the 48-hour test. The frequency of occurrence of primary oogonia was significantly ($p < 0.05$) lower in atrazine-exposed (43.7%) tadpoles relative to controls (74%); however, the frequency of occurrence of secondary oogonia was significantly ($p<0.05$) higher in atrazine-exposed (36%) tadpoles compared to controls (23%). The incidence of atretic primary and secondary oogonia was significantly higher ($p < 0.05$) in atrazine-exposed ovaries (20.2%) relative to control (2%). Furthermore, sections of the pituitary revealed no histological evidence that the pituitary was actively secreting hormones. The authors concluded that atresia could reduce the reproductive capacity of the tadpole since primary germ cells provide oocytes for all the subsequent cycles of oogenesis in the reproductive life of the frog. The authors also speculated that atrazine may be affecting aromatase activity; however, it was uncertain whether enhanced conversion of androgens to estrogen could provide the effects observed.

Similar to the evaluation of Tavera-Mendoza *et al.* (2001*a*), this study provides useful information on hazard identification and measurement endpoints, such as gonadal abnormalities. However, this study also contained uncertainties concerning use of a single dose, the nature of the measurement endpoints, and the relevancy of the endpoint to higher order measures of organismal fitness.

Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. A Pilot Study of Response of Larval Rana clamitans to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology and Selected Hormones and Enzyme Activities. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-03. (EPA MRID No. 458677-03)

The objectives of this study were to develop and validate culture and test methods for conducting laboratory studies with *Rana clamitans* and to determine the response of larval *R. clamitans* to atrazine by assessing metamorphosis and reproduction indices when animals were exposed to 10 and 25 µg/L during larval development. In this study the following indices were evaluated: 1) percent of larvae initiating metamorphosis, 2) percent completing metamorphosis, 3) time to metamorphosis, 4) fresh post-mortem body weight and snout-vent length, 5) incidence of gross gonadal abnormalities, and 5) histology of the gonads. Beginning five-days post-hatch, green frog tadpoles reared from field-collected eggs were exposed to atrazine for 273 days. Positive controls, dihydrotestosterone and 17-β estradiol (0.1 µg/mL in 0.005% ethanol), a dilution water control and a solvent control (0.005% ethanol), were also included. Replicates (9) consisted of 30 free-swimming tadpoles each. From exposure days 0 - 67, animals were maintained under static renewal conditions in 4 L of test solution, with 50% tank changes conducted every 72 hrs. From days 68 to 273, tadpoles were maintained in tanks containing 16L of test solution under static renewal. After 273 days, exposures were terminated and tadpoles were maintained in continuous flow-through 10-L glass tanks housed in large acrylic tanks containing 80 L of continuously renewing freshwater.

At fore-limb emergence, tadpoles were either housed individually or in small groups in 10-L glass tanks containing approximately 500 mL of fresh water. Over the study period, mortality across all treatment groups averaged 76.5%, which was attributed to poor water quality and overcrowding during the 273-day static-renewal phase of the study. Mean-measured concentrations of atrazine were relatively consistent with nominal values, but these measurements were made on freshly prepared stock solutions, which made it difficult to determine atrazine concentration in aged exposure solutions. Measurable levels of atrazine were detected in the dilution water controls. Although the concentrations of positive control hormones were not

measured, the positive control treatments (dihydrotestosterone and 17-β estradiol) suggested that green frogs only reacted to androgenic chemicals, skewing the sex ratio to predominantly male frogs (97.6%). It is uncertain if these results indicate that green frogs were unresponsive to estrogenic chemicals or whether there was insufficient estradiol in solution to elicit an effect. Based on gross morphology, no hermaphroditism (testicular and ovarian tissue in the same animal) was observed in any of the treatment groups. There were, however, difficulties in discerning the presence of gonads using this process. At the lower dose of 10 µg/L atrazine, time to and age at metamorphosis and the size of metamorphs were reduced in frogs, while at the higher dose of 25 µg/L atrazine, there was no difference in these same parameters.

From a design standpoint, results from this study were difficult to interpret because only two concentrations of atrazine were tested, comparatively few frogs survived to complete metamorphosis, gonadal histology and aromatase levels were not provided, and only one tank per treatment level was used. The means to establish a dose-response relationship was also limited by the use of two exposure concentrations. Furthermore, the high mortality noted in this study may be indicative of poor water quality and overcrowding. The lack of response to the positive estradiol control and the presence of atrazine in the dilution water control may have confounded interpretation of the data and suggests that the study design may not be adequate to evaluate the potential effects of atrazine on green frogs. Alternatively, the species used in this study may not be a sensitive model for examining the effects of atrazine on amphibian development.

Villeneuve, D. L., K. Coady, M. Hecker, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Methods development for the study of mechanism of action of atrazine in adult and metamophosing *Xenopus laevis* and *Rana clamitans*: aromatase induction. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-01 (EPA MRID No. 458677-08)

In this study, frogs were exposed to nominal atrazine concentrations of 25 µg/L to test whether the pesticide could up-regulate aromatase activity in sexually mature male and female *X. laevis* and to evaluate whether atrazine would decrease plasma testosterone and increase estradiol (consistent with an increase in aromatase activity) in frogs. In three separate studies, two

involving adult males and one with adult females, frogs were exposed either to atrazine or to fresh water under static renewal conditions with 50% exposure solution changes every 72 hrs (single tank per treatment). In the first study, 15 males were exposed for 26 days. In the second study, 15 males were exposed for 43 days (single tank per treatment), and in the third study 13 females (six in one replicate and seven in the second replicate) were exposed for 47 days. Overall mortality was 3, 7, and 19% in the 26-, 43- and 47-day exposures, respectively; mortality was primarily associated with disease (fungal/bacterial) and was positively correlated (r=0.77) with the number of exposure days.

Homogenates from a single testes and from brain were used to measure aromatase activity in males. Aromatase activity in the testes was at or near the level of detection (LOD = 0.025 fmol/h/mg protein). After 26 days of exposure, mean brain aromatase activity from atrazine-exposed males (8.4 ± 4.2 fmol/h/mg protein) was not statistically different ($p = 0.678$) from the controls (7.1 ± 4.2 fmol/h/mg protein). Following 43 days of exposure, mean brain aromatase activity in atrazine-treated males (5.8 ± 3.4 fmol/h/mg protein) again was not statistically different ($p = 0.199$) from the controls (10.4 ± 7.1 fmol/h/mg protein). In the second study, atrazine concentrations as high as 0.25 µg/L were measured in the dilution water control. In the third exposure with female frogs, ovarian aromatase activity averaged 4.5 ± 1.7 fmol/h/mg protein and did not differ statistically ($p=0.447$) from controls (5.4 ± 2.1 fmol/h/mg protein). Mean aromatase activity of brain homogenates from atrazine-exposed females was 7.3 ± 5.0 fmol/h/mg protein, and did not differ significantly ($p= 0.582$) from control females (8.9 ± 4.2 fmol/h/mg protein).

Tank effects were difficult to document in the first two studies because only one tank was used per treatment level. There also was considerable variability in aromatase activity between frogs receiving the same treatment, with coefficients of variability at or exceeding 100%. Other confounding factors included the use of only one atrazine concentration and atrazine contamination of the controls in the second study.

The authors recommended that future testing use replication, higher sample sizes and a broader range of atrazine levels to test for potentially subtle effects. Although not discussed in the report, water quality may have also compromised the study. Evidence in support of this

includes the correlation of mortality with the number of days the frogs were confined and the references to the diseased state of the dead animals. Apparently, *Xenopus* are susceptible to bacterial septicemia (red-leg disease) if poor water quality persists (Sive *et al.* 1998).

Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Response of *Xenopus laevis* to Atrazine Exposure: Assessment of the Mechanism of Action of Atrazine. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-04.(EPA MRID No. 458677-04).

The goal of this study was to determine the effects of atrazine on metamorphosis and reproductive indices of larval *X. laevis* exposed from 72 hrs after hatch until completion of metamorphosis. Indices evaluated at metamorphosis included percentage of animals initiating metamorphosis, percentage of animals completing metamorphosis, time to metamorphosis, fresh body weight, snout-vent length, size of the laryngeal dilator muscle, and gonad development. Additionally, the study was designed to determine concentrations of circulating hormones, including testosterone and estradiol, in control and atrazine-treated *X. laevis* and to measure aromatase activity in the gonads and brain tissue of control and atrazine-exposed *X. laevis*.

In this study, *X. laevis* larvae were exposed to atrazine at nominal concentrations of 0.1, 1.0, 10, and 25 µg/L in frog embryo teratogenesis assay–*Xenopus* (FETAX) media (a moderately hard reconstituted water) using a static renewal system where 50% of the exposure solutions were changed every 72 hrs. Exposures were also conducted using dilution water, positive (0.1 µg/L17-β estradiol and 0.1 µg/L dihydrotestosterone) and solvent (0.005% ethanol) controls. Larvae were exposed from 72-hrs post-hatch through metamorphosis (NF stage 66). At metamorphosis, a subset (number not reported) was euthanized; gonads were examined for gross morphology, and gonads plus the larynges were prepared for histology. The remaining animals were exposed until 2-3 months post-metamorphosis. After the exposure period, half the frogs were examined for gross morphology and the other half (50 frogs per treatment) examined for histology of the gonads. One frog from each replicate tank (64 frogs total) was randomly selected, and blood (drawn by cardiac puncture), brain, and gonads were collected for sex steroid hormone and

aromatase activity assays. Plasma concentrations of testosterone and estradiol were measured by enzyme-linked immunosorbent assay (ELISA), while a tritium-labeled androstenedione water release assay was used to measure aromatase in brain and gonad tissue.

The authors concluded that atrazine treatment did not affect mortality, time to metamorphosis, sex ratio, gonadal development, aromatase activity or steroid hormone plasma concentrations in a dose-dependent fashion. They also concluded that estradiol (positive control) treatment only appeared to increase estradiol plasma concentrations. Dihydrotestosterone (positive control) increased larynx dilator muscle area in females, and neither positive control influenced sex ratios.

Although the most frequent gonadal abnormality based on gross morphology was discontinuous gonads, histology indicated that mixed sex/intersex (ovarian and testicular tissue in the same frog) was much more common than indicated by gross morphology. Since histology is still being conducted, the authors could not conclude that gonadal abnormalities were treatment-related.

Poor water quality (elevated ammonia and nitrite with low dissolved oxygen) probably resulting from relatively high loading rates (30 tadpoles/4 liters of exposure solution) under static conditions may have compromised the growth and development of the test animals. On average, it took 73 days for frogs to complete metamorphosis, as opposed to 58 days reported by Nieuwkoop and Faber (1994), and 17 (<2%) frogs in the study never underwent metamorphosis. The dilution water controls had measured atrazine concentrations of 0.1 µg/L. High variability (coefficients of variation ranging as high as 524%) associated with gonadal aromatase activity and with plasma steroid hormone concentrations made it difficult to differentiate treatment effects. Also, estradiol treatment failed to skew sex ratios significantly in favor of females as expected. In conclusion, a combination of tank effects, contaminated controls, prolonged development time, high variability, and lack of responsiveness to estradiol limited the usefulness of this study in differentiating treatment effects.

Goleman, W. L. and J. A. Carr. 2003. Response of larval *Xenopus laevis* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology. The Institute of Environmental and Human Health, Texas Tech University, Texas Tech University Health Sciences Center, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number TTU-01 (EPA MRID No. 458677-07).

Although this study also appeared recently in *Environmental Toxicology and Chemistry* (Carr *et al.* 2003), the raw data for this study were submitted to EPA by the registrant allowing a more detailed evaluation. The goal of this study was to determine the response of larval *X. laevis* to atrazine by assessing metamorphosis and reproductive indices when animals were exposed from 48 - 72 hrs after hatching through the completion of metamorphosis. Indices evaluated included percent of animals initiating metamorphosis, percentage of animals completing metamorphosis, time to metamorphosis, percentage of intersex gonads, fresh post-mortem body weight, snout-vent length, and laryngeal size.

In a 78-day exposure, 48- to 72-hr post-hatch *X. laevis* larvae were exposed to nominal concentrations of 1, 10 and 25 µg atrazine/L in FETAX medium, FETAX medium alone (dilution water control), 0.1 µg/L 17-β estradiol, 0.1 µg/L dihydrotestosterone, or solvent control (0.0025% ethanol) in a static renewal system where 50% exposure solution water changes occurred every 72 hrs. For the first seven days, 60 - 65 larvae were maintained in 100 mL of exposure solution. At day 14, animals were transferred to 1 L of exposure solution, and by Day 21, animals were maintained in 4 L of exposure solution. At NF Stage 66 animals were weighed, measured for snout-vent length and examined for gonadal gross morphology. Larynx and gonads also underwent histological analysis.

Mortality over the study period ranged from 10 -14% for those animals that reached stage 66 by 80 days post-hatch. Time to complete metamorphosis did not differ significantly across treatments although the specific time was not reported. In all treatments, weight and snout-vent length were inversely proportional to the number of days required to complete metamorphosis, *i.e.*, animals completing metamorphosis early tended to be larger than animals that took longer to complete metamorphosis. Sex ratios ranged from 48 - 50% male across all treatments except for the estradiol treatment, which significantly skewed the ratio in favor of females (67%; statistical

significance not reported). While the incidence of intersex was significantly correlated ($p = 0.0003$) with atrazine exposure concentrations, only the 25 µg atrazine/L (4.7%) and estradiol-treated (7.4%) males had incidence rates significantly different ($p = 0.0061$ and $p=0.01$, respectively) from dilution water (0.6%) and solvent (0.0%) controls. Intersex in males treated with 25 µg atrazine/L was characterized by distinguishable testicular and ovarian tissue, while males treated with estradiol sometimes had ambiguous tissue structures.

There was no difference in the cross-sectional area of larynx dilator muscle in atrazine-treated males relative to dilution water controls. Dihydrotestosterone-treated females had significantly larger ($p < 0.0001$) cross-sectional dilator muscle areas than solvent control females.

In this study, atrazine did not impact length, weight, time to metamorphosis or dilator muscle area relative to the controls; however, exposure to 25 µg atrazine/L appeared to significantly increase the number of intersex males and animals with discontinuous gonads. The observation that both body weight and length were inversely correlated with the length of time to complete metamorphosis suggested that animals in all treatment groups were developmentally delayed. In addition, 17-β estradiol treatment only resulted in 67% females, further suggesting that study animals may not be totally responsive to the positive control. Reported dissolved oxygen concentrations did not drop below 3.9 mg/L; however, ammonia levels as high as 27 mg/L were reported. These high levels of ammonia suggested that the 50% static renewal and loading rates (number of tadpoles per liter of exposure solution) may have resulted in poor water quality, which could have slowed development of the animals. In support of this possibility, roughly 42% of the animals did not appear to have reached stage 66 by Day 78, suggesting that a large proportion of the animals were not developing at a normal rate. Given the declining condition of the frogs with increased length of time to maturity, it is unclear whether these animals would have metamphosed and/or survived. Because all the animals in the study had not undergone metamorphosis, the percent initiating metamorphosis, time to metamorphosis and percentage of gonadal abnormalities cannot be accurately calculated relative to the total animals used in the study.

In the context of hazard identification, Goleman and Carr (2003) provides additional information that atrazine exposure could potentially cause gonadal developmental effects in

amphibians, albeit at a much higher concentration than reported by Hayes *et al*. (2002*a*; ≥0.1 ug/L) but comparable to the concentration reported by Tavera-Mendoza *et al.* (2001 a,b; 18 ug/L). However, Goleman and Carr (2003) did not report effects on secondary sexual characteristics, *i.e*. laryngeal muscle diameter, as observed in other amphibian studies (Hayes *et al.*, 2002*a*).

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002*b*. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. ***Environmental Health Perspectives***. http://dx.doi.org/ *[Online 23 October 2002]*

Because components of this study were conducted both in the laboratory and in the field, EPA has described its review in both sections of the document. The laboratory phase of the study is discussed here, while the field component of the investigation is discussed in the next section of this paper.

The objective of this study was to investigate whether gonadal effects observed in previous studies of non-native *X. laevis* exposed to atrazine were also exhibited in a native species, *i.e., R. pipiens.* Leopard frog larvae were exposed from 48-hrs post-hatch through complete tail resorption (NF-Stage 66) to nominal atrazine concentrations of 0.1 and 25 µg/L (0.0036% ethanol) in 10% Holtfreter's solution. Following exposure, animals were sacrificed. Gross morphology and histological analysis of gonads revealed that 36% and 12% of the males treated with atrazine at 0.1 and 25 µg/L, respectively, suffered from gonadal dysgenesis (under-developed testes with poorly structured, closed lobules and low to absent germ cells). Further, 29% of the 0.1 µg/L and 8% of the 25 µg/L animals displayed varying degrees of sex reversal; testicular lobules of sex-reversed males contained oocytes, and males that metamorphosed later contained large numbers of oocytes. In a few cases, testicular oocytes were reported to be vitellogenic, *i.e.*, to contain yolk.

The study suggested that the enhanced response at lower doses may be consistent with the low-dose effect reported for certain endocrine-disrupting chemicals although the data did not show a clear dose-response relationship. In a previous study using *Xenopus* (Hayes *et al.* 2002*a*),

there appeared to be a threshold effect for testicular abnormalities at 0.1 µg/L atrazine (nominal); however, the response appeared to remain steady across concentrations of atrazine up to 200 µg/L, with 16 - 20% of the males exhibiting gonadal abnormalities. However, the incidence of these changes at each dose was not reported. As a result, the authors concluded that an "inverted-U" (parabolic) dose-response curve appropriately described the observations.

This study essentially replicates previous work conducted by Hayes *et al.* (2002*a*) that demonstrated gonadal developmental effects in *X. laevis* and thus provides additional hazard identification data on potential effects in the native leopard frog. However, because the study employed two atrazine concentrations (nominal concentrations of 0.1 and 25 ug/L), there is not sufficient information to establish a dose-response relationship.

Field Studies

In addition to the laboratory studies, EPA evaluated a series of field studies ranging from pilot reconnaissance surveys to full multi-year investigations. While field studies may provide useful insights on "real-world"responses and reflect what may actually occur in a natural setting, they can be subject to a wide range of environmental effects that can influence a study's measurement endpoints beyond treatment effects. Unlike controlled laboratory studies where environmental conditions and test animal homeostasis are artificially maintained to some extent, field studies are subject to a wide range of conditions that can influence the fitness of organisms being studied. In addition, under natural conditions, organisms are potentially exposed to a wide range of chemical and non-chemical stressors simultaneously, which makes the elucidation of cause-effect and dose-response relationships difficult to establish. Studies measuring the effects of chemical exposures must allow for the potential presence of a wide range of chemicals in the field and the possibility that these chemicals may have interactive effects that could be antagonistic, additive or synergistic. In addition, the presence of non-chemical stressors (*e.g.*, suspended solids) can modulate or attenuate potential chemical exposures or effects. Thus, while field studies provide an opportunity to understand how laboratory effects may be expressed under more natural conditions, these studies may introduce a range of interpretation challenges that

need to be considered. Additionally, the wide range of conditions may increase the variability associated with measurement endpoints, making it difficult to detect treatment effects. For these reasons, variability caused by non-treatment related environmental parameters should be controlled or accounted for through study designs that include a sufficient characterization of field conditions to permit statistical analyses of direct, indirect, and interactive effects and typically employ large sample sizes.

EPA evaluated a total of ten field studies consisting of two open literature studies and eight Syngenta-sponsored studies. Although a South African study of *X. laevis* was a composite of four studies, it was reviewed collectively as a single study. In two of the field studies, part or all of the study was conducted under relatively controlled conditions either in the laboratory or in an outdoor microcosm. The field studies were conducted on a range of species, *i.e.*, cricket frogs (*Acris crepitans*), *X. laevis*, R. clamitans , bull frogs (*Rana catesbiena*), and cane toads (*Bufo marinus*) in a wide range of locales (Florida, Indiana, Iowa, Michigan, Nebraska, Utah and Wyoming, USA, and South Africa). Each of the studies was conducted in the native range for the species and in many cases the studies represented interim reports which were the first year summary of a planned three-year study.

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002*b*. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. ***Environmental Health Perspectives*** http://dx.doi.org/ *[Online 23 October 2002]*

As discussed earlier, this investigation included two components: a laboratory phase and a field phase. Field studies were initiated along with laboratory studies in order to address the ecological significance and relevance of the initial laboratory studies discussed previously. The objective of the field component phase of the study was to determine if the effects of atrazine on leopard frogs observed under controlled laboratory conditions could also be observed in wild *R. pipiens* from a variety of habitats in areas with reportedly low- and high-atrazine use. In this field study, water samples were collected at each site to determine atrazine exposure.

In the field reconnaissance survey of leopard frogs, frogs were sampled from a total of eight sites with the assumption that low- and high-atrazine sales data would be indicative of low- (four sites) and high- (four sites) atrazine exposures. Aqueous atrazine concentrations were quantified by gas chromatography/mass spectrophotometry with a level of detection of 0.1 µg/L in 100-mL water samples at each of the eight sampling sites. It should be noted, though, that in this study it was difficult to determine if atrazine concentrations reflected the true exposure conditions during larval development because the study did not include information on when the animals underwent metamorphosis. Field collection of frogs and water samples proceeded eastward, starting in Utah on July 15, 2001 and ending in Iowa on July 28, 2001. At each site, the researchers collected 100 leopard frogs for histological analysis and 100 mL of water for chemical analysis.

The researchers identified testicular oocytes in males from seven of the eight collection sites. All sites with atrazine levels exceeding 0.2 µg/L had males that displayed sex-reversal similar to those abnormalities induced by atrazine in the laboratory study. The highest incidence (92%) and most advanced cases of hermaphroditism were observed in animals collected from the North Platte River in Wyoming where the measured atrazine concentrations were lower than at most of the other sites. At sites with similar atrazine concentrations, *e.g.*, 0.2 µg/L, the incidence of gonadal abnormalities ranged from 9 to 92% suggesting that there was no clear pattern of response. At sites where no atrazine was found, the incidence of testicular oogenesis appeared to be as high as 18%.

This study was useful in identifying field effects similar to those observed in laboratory studies of the leopard frog. Like the laboratory study, this study was unable to establish a quantitative dose-response relationship. Notably, atrazine was often measured where it was not reportedly used. Moreover, it is likely that other chemical contaminants were also present but not measured. Although the researchers conducted pesticide residue analyses for chemicals that were reportedly used in the watershed, the scope of the chemical analyses varied on a site-by-site basis. Information concerning the chemical profile and morphological characteristics of the sampling sites needs to be provided in order to determine the comparability of the associated aquatic ecosystems.

DuPreez, L. H., A. M. Jooste and K. R. Solomon. 2003. Exposure of *Xenopus laevis* larvae to different concentrations of atrazine in semi-natural microcosms. School of Environmental Sciences and Development, Zoology Department, Potchefstroom University fo CHE, Potchefstroom 2520, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01-D (EPA MRID No.458677-11).

This report represents an interim summary of a longer-term investigation. The objective of this study was to assess the effects of atrazine exposure on gonadal abnormalities in *X. laevis* metamorphs and sub-adults under semi-natural conditions in microcosms. Based on the absence of atrazine use and corn production in the watershed and the presence of *X. laevis*, adult frogs were collected from a Potchefstroom, South African earthen pond C6, which was considered to be reference site. Adults were induced to spawn in the laboratory and the 96-hr old progeny were divided among four nominal treatments (0, 1, 10 and 25 µg/L atrazine) with three replicates per treatment (800 tadpoles per replicate). Mean measured concentrations of atrazine ranged from 0.9 to 1.82 µg/L, 10 to 15.9 µg/L, and 23.8 to 39.7 µg/L for the 1, 10, and 25 µg/L nominal treatments, respectively. Atrazine in the reference tanks ranged from 0 to 0.1 µg/L. Microcosms consisted of 1600-L cement tanks, lined with polyethylene with a 3-cm sand bottom, and filled with 1,100 L of tap water. Larvae were exposed until they reached NF stage 66; the study was terminated after 133 days of exposure. A total of 150, NF stage 66 frogs were sampled per treatment. Snout-vent length, weight, days to NF stage 66 were recorded and animals were examined for gross gonadal morphology.

A series of figures depicted water temperature, pH, dissolved oxygen (DO) and conductivity over the study period. Dissolved oxygen concentrations fluctuated from 1 to 8.0 mg/L over the course of the study. From day 90 to 100, conductivity, temperature and pH all dropped markedly. Conductivity in most of the exposure tanks tended to increase from days 0 to 90; however, tanks 2,4, 6 and 9 had erratic conductivity with marked declines. In tanks 4 and 6, conductivity dropped by roughly 40% on day 40, and in tank 2 conductivity declined by a similar amount by day 60.

Although some animals reached stage 66 by 70 days, most did not reach metamorphosis until 126 - 133 days. *Xenopus laevis* tadpoles took 58 days to complete metamorphosis under controlled laboratory conditions of 20 - 25°C (Nieuwkoop and Faber 1994). Under natural conditions, where water temperatures may fluctuate widely, metamorphosis may take from 56 to 63 days, which is still a shorter period of time than the 70 days in the current study. The authors attributed low water temperatures to delayed development of the tadpoles. Based on gross morphology, the incidence of gonadal deformities in 1, 10 and 25 µg/L atrazine groups was 1.3, 0.7 and 3.3% of the total frogs examined (150), respectively; reference frogs exhibited a 4% incidence of gonadal deformities. Discontinuous testis was the only gonadal abnormality identified; no abnormalities were observed in the ovaries except for one ovary that was reduced in size. Males comprised 48, 39, and 47% of the 1.0, 10, and 25 µg/L atrazine-treated samples, respectively, while the reference samples were 45% male. In addition, male and female frogs not exposed to atrazine were larger, in terms of length ($p<0.03$) and weight ($p<0.02$), than their atrazine-treated counterparts.

This interim microcosm study represents a reasonable step forward in testing for effects observed in the laboratory and may provide useful information when all the analyses are completed. The present study, though, showed that developmental rates across all treatments, in terms of time to metamorphosis, were delayed. Fluctuating water quality in the microcosm units may have impacted the developmental rate. Additionally, the study was designed with the assumption that phytoplankton growth would serve as a source of food for developing tadpoles. Although the authors note that phytoplankton "flourished", no measure (*e.g.*, chlorophyl *a* concentration) of phytoplankton growth was reported and supplemental food (rabbit pellets) had to be provided to the developing tadpoles. However, phytoplankton growth could have been significantly limited by atrazine treatment, thereby resulting in indirect effects of atrazine on development. In support of this possibility, atrazine-treated male and female frogs were significantly smaller in terms of length and weight than their "untreated" counterparts, further complicating interpretation of the study results. This assessment was based on gross morphology alone, and the results may change when histology is completed.

Reeder, A. L., G. L. Foley, D. K. Nichols, L. G. Hansen, B. Wikoff, S. Faeh, J. Eisold, M. B. Wheeler, R. Warner, J. E. Murphy, and V. R. Beasley. 1998. Forms and Prevalence of Intersexuality and Effects of Environmental Contaminants on Sexuality in Cricket Frogs (*Acris crepitans*). *Environmental Health Perspectives* 106 (5): 261 - 266

To assess the prevalence of gonadal abnormalities in adult and juvenile cricket frogs, and to determine whether sexual development is altered in response to exposure to environmental contaminants, cricket frogs were collected over a three-year period (1993 - 1995) in various locations throughout the state of Illinois. Additionally, water/sediment samples were collected at sampling sites in 1994 and 1995 to determine whether the prevalence of intersex could be related to chemical residues. In a separate study, cricket frogs were collected at a site known to be contaminated with PCBs and PCDFs, and the prevalence of intersex was determined relative to control sites.

Of the 55 adult and juvenile male and female frogs collected in 1993, two (3.6%) had both an ovary and testis. In the testis of one, spermatogenesis was normal; in the other, an immature ovary was present as well as a testis with no active spermatogenesis. Of the 243 frogs examined in 1994, six (2.5%) contained both an ovary and a testis, and five of the affected animals had areas of normal spermatogenesis in the testis interspersed with oocytes. One animal had a mature ovary and mature testis with normal spermatogenesis. Of the 43 frogs examined in 1995, only one (2.3%) had an ovotestis. Across all three sampling years the prevalence of intersex was 2.8%. In specimens with an ovary on one side and a testis on the other, ovarian size ranged from well-developed mature female size to extremely small ovaries with a few oocytes present.

Of the five sites where intersex organisms were found, four had detectable atrazine (limit of detection: 0.5 µg/L). Of the four sites where no intersex organisms were collected, only one contained detectable levels of atrazine. According to the authors, the relationship between detection of atrazine and prevalence of intersex approached significance ($p = 0.07$). At one site treated with copper sulfate in 1994, one out of 33 frogs collected had an ovotestis. In 1995, no relationship was found between the detection of atrazine and the prevalence of intersex. No intersex was identified in frogs collected from a pond treated with endothall in 1995. Concentrations of lead from both years were not associated with intersex.

Of the frogs collected from PCB and control sites, one frog with an ovotestis was identified from the control site. Sex ratios differed significantly (probability not given) between contaminated and control sites. In 13 juveniles from control and 13 from contaminated sites, gonadal tissue was immature and could not be identified for histological preparation. The association between sex ratios of PCB/PCDF contaminated and control groups revealed a significant difference ($p = 0.0007$).

While a wide range of chemical residue analyses were conducted, only atrazine data were reported. The authors suggested that there may be a trend between atrazine and the proportion of animals exhibiting intersex; however, the only statistically significant relationship was between sex ratios in PCB/PCDF contaminated sites relative to controls. It should be noted that the sample size for making this determination was low, with three control and three contaminated site animals.

In this paper, Reeder *et al.* (1998) discussed the range of chemical residues in the field collection sites and how these chemicals, combined with environmental conditions, could impact gonadal development. These factors contributed to the limited utility of this study because the investigation did not demonstrate a significant effect of chemical residues on the prevalence of intersex in cricket frogs. This study underscored the need to have focused study designs with sufficient power in terms of sample size to discriminate effects if they exist. Also, the report acknowledged that little is known about natural intersex rates in cricket frogs. Without a better understanding of the biology of the cricket frog and the toxicological phenomenon being examined, it is difficult to interpret the significance of the reported observations.

South African *X. laevis* Field Studies:

Smith, E. E., L. DuPreez, and K. Solomon. 2003. Field exposure of Xenopus laevis to atrazine and other triazines in South Africa: feasibility study for site characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, Texas (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01A.

Smith, E., L DuPreez and K. Solomon. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: exposure characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental & Human Health, Texas Tech University, Lubbock, Texas 79490 (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Private Bag X6001, Potchefstroom 2520 (South Africa). Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01B

Smith, E. E., L. DuPreez, and K. Solomon. 2003. Gonadal and laryngeal responses to field exposure of *Xenopus laevis* to atrazine in areas of corn production in South Africa. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01C.

Giesy, J. P., M. Hecker, and P. D. Jones. 2003. South African Analytical Support – Hormone and Aromatase Analysis (SA- 01C). Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-07

The objective of these studies was to examine the effects of atrazine on *X. laevis* in its native habitat (South Africa). Initially these studies were intended to test whether morphological and biochemical differences existed between clawed frogs in atrazine-exposed (experimental) versus non-exposed (reference) ponds. The criteria for differentiating reference and experimental sites included production of corn and use of atrazine in the vicinity, plus the presence of *X. laevis* in a pond. Based on an initial survey of the sampling area, five experimental (atrazine exposure)

and three reference (no atrazine exposure) sites were selected (458677-09). However, subsequent sampling during later phases of the study revealed that the reference sites all contained measurable residues of atrazine, its degradates, and terbutyhylazine (triazine herbicide not registered for use in the USA) that were, in some cases, higher than sites considered representative of atrazine exposure (458677-01).

The study sites were subject to unusual conditions, including abnormally high rainfall, extremes in pH (10.2 - 10.8), and shifts in the natural mortality rate due to predation by the introduction of sharptooth catfish (*Clarius gariepinus*). Although other pesticides besides herbicides were used at these sites, no data were presented to characterize these residues. Monthly terbuthylazine residues were higher at some reference sites than at experimental sites, and some reference ponds had unusually high amounts of chromium in the soil (100 mg/kg) and titanium in the water (0.6 mg/L) (458677-01). Depending on the form in which these metals were in, chromium and titanium concentrations at these levels exceeded the reported LC_{50}s for these metals in Fowler's toad, *Bufo fowleri*, and the Eastern narrow-mouth toad, *Gastrophryne carolinenis* (Birge *et al.* 2000) by several orders of magnitude.

In this study, fewer frogs were targeted in high atrazine exposure (atrazine concentration range: 1.46 - 11.6 µg/L) sites compared to low atrazine exposure (atrazine concentration range: 0.41 - 1.62 µg/L) sites, and there was difficulty in obtaining even the reduced number of test animal at experimental sites. For this reason, the animals had to be collected over an extended period of time (up to 6 months). The collection method (traps baited with meat scraps) may have placed adult male and females in very close proximity to one another for 48 hrs. As opportunistic breeders, *X. laevis* held in close proximity to one another may undergo marked changes in sexual readiness. The collection method may have also provided an exogenous source of steroid hormones via the feed. Studies have shown that ground beef liver can produce growth inhibition and abnormalities in *X. laevis* larvae (Nieuwkoop and Faber 1994).

The authors concluded that there were no differences in the lengths or weights of either males or females collected from reference (low atrazine) and experimental (high atrazine exposure) sites although, at both sites, females were larger than males. Testes from frogs collected at high atrazine sites tended to weigh more than testes collected from frogs at reference

sites, and the gonadosomatic index (ratio of gonad weight to total body weight) tended to be higher for males at experimental sites (458677-10).

Testicular oocytes were observed in 3% of the reference frogs and in 2% of the frogs from experimental sites. No other significant morphological differences were observed in the testes collected from experimental and reference sites. (458677-10)

Males from ponds with the highest atrazine concentrations had significantly lower plasma median testosterone concentrations than males from reference sites, and log male testosterone levels were negatively correlated ($r=^{-}0.839$; $p=0.009$) with the log of the atrazine degradate, diaminochlorotriazine concentration. Females collected at high atrazine exposure sites had significantly higher ($p=0.018$) testosterone levels than females collected at reference sites; female testosterone levels were negatively correlated with atrazine surface water concentrations (458675-01). Similarly, plasma estradiol concentrations were lower in males and females collected from high atrazine sites. Male estradiol concentrations were negatively correlated ($r=^{-}0.779$; $p=0.023$) with diaminochlorotriazine (DACT), while female estradiol concentrations were negatively correlated ($r = ^{-}0.833$; $p = 0.010$) with concentrations of the parent atrazine (458675-01).

In this study, aromatase activity in testes could not be quantified at all sites; thus, no comparisons were made between low and high atrazine exposure sites. Ovarian aromatase activity was not significantly different between sampling sites. The highest amount of variability in aromatase was observed in animals collected over a protracted period of time, which underscores how the extended collection period influenced the results. The current data suggest that atrazine and/or its degradates may impact plasma testosterone; however, the data are not conclusive and the mechanism underlying this phenomena cannot be identified.

Since atrazine was present to some degree in all of the sampling sites, it was difficult to test a null hypothesis in which atrazine was assumed to be absent in the control group. The presence of atrazine in all of the ponds suggested that a regression based approach to data analysis may be appropriate, but this type of analysis would require a substantially different experimental design for optimal power to differentiate treatment effects. Of course, EPA

recognizes that controlling critical parameters and reducing confounding effects is one of the major difficulties facing the conduct of all field studies.

Jones, P. D., M. B. Murphy, M. Hecker, J. P. Giesy. 2003. Tissue Pesticide Residues and Histology of the Larynx and Gonads in Native Green Frogs (*Rana clamitans*) Collected from Agricultural Areas in Michigan: Hormone Analysis. Aquatic Toxicology Laboratory, Michigan State University, 218C National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc. Laboratory Study ID: ECORISK Number MSU-02 (EPA MRID No. 458677-02).

The objective of this study was to assess the effects of atrazine on kidney and gonad histology and plasma steroid hormone concentrations and gonadal aromatase activity of green frogs (*R. clamitans*) and other incidental ranid species collected from various field sites within their native Michigan ranges. For Phase I of a three-year study, *R. clamitans*, *R. pipiens*, and *R. catesbeiana* were collected from three reference ponds (atrazine concentration range: 0.015 to 0.093 µg/L) and six atrazine-exposed ponds (atrazine concentration range: 0.025 to 250 µg/L) in Michigan. Both juvenile (372) and adult (340) green frogs were collected and examined for gross gonadal abnormalities, and blood plasma estradiol and testosterone levels were measured. A total of four mixed or unknown sex animals were identified in all of the frogs collected. Because hormone levels exhibited considerable variability among locations and individuals, relationships between plasma hormone levels and atrazine exposure could not be determined.

While this study represents an interim report, the preliminary results suggested that *R. clamitans* was not markedly impacted by atrazine exposure in terms of gonadal deformities. Because the reference sites may have contained atrazine, ecological effects could not be accurately discriminated. Additionally, plasma testosterone and estradiol levels exhibited considerable variability, and differences between “exposed” and “reference” sites could not be detected. Plasma steroid levels were of questionable utility, though, since data were collected over four months in adult frogs, and coefficients of variation ranged as high as 10,628%. In this study, there were no gender-specific differences in plasma estradiol levels. Exposed males contained similar (roughly 90%) amounts of estradiol as females, while at the reference sites males exhibited roughly 5.0 times the plasma estradiol concentration than females.

Because of the variability associated with the measured plasma steroid hormone levels and the presence of low levels of atrazine at all the sites, this study has limited usefulness in distinguishing any treatment-related effects in amphibians.

Sepulveda, M. S. and T. S. Gross. 2003. Characterization of Atrazine Exposures and Potential Effects in Florida Ecosystems Dominated by Sugarcane Agriculture: A Reconnaissance Survey of Amphibians in South Florida for the Assessment of Potential Atrazine Effects. Department of Physiological Sciences, University of Florida, Caribbean Science Center, Gainesville, Florida. Sponsor: Syngenta Crop Protection, Inc. Study ID: ECORISK Number UFL-02 (EPA MRID No. 458677-06)

The goal of this study was to determine whether exposure of toads to atrazine in sugarcane agricultural areas in south Florida could result in a higher incidence of intersex and/or other gonadal/developmental anomalies. Secondarily, the study was conducted to examine amphibian populations exposed to a complex mixture of agrochemicals, *e.g.*, insecticides, herbicides, fungicides and fertilizers. This reconnaissance survey for cane toads in the southern Florida sugarcane-dominated, agricultural sites (Belle Glade and Canal Point) and in nonagricultural sites (University of Miami) indicated an increased incidence of intersex (ovarian tissue in Bidder's organ) in toads identified as having testes. Approximately 29% of the males collected from Belle Glade and 39% of the males collected from Canal Point were intersexed, while no intersex frogs were identified among the University of Miami samples. *Bufo marinus* is typically a sexually dimorphic amphibian; however, 100% of the cane toads collected at Belle Glade and roughly 55% of the male cane toads collected at Canal Point exhibited female coloration. Additionally, males typically exhibit nuptial pads, but in this study 71% and 0% of the intersex toads collected from Canal Point and Belle Glade, respectively, had nuptial pads. Vitellogenin, a female-specific protein, is not typically expressed in males; however, intersex toads had vitellogenin levels (774 ± 29 PO_4/mg protein) similar to the females (853 ± 34 PO_4/mg protein) and was roughly double that of male toads (375 ± 34 PO_4/mg protein) collected from the nonagricultural site. Although plasma sex steroids (17-β estradiol and testosterone) were relatively gender-specific; testosterone levels in intersex males exhibited roughly twice the amount of variability as similar estimates for males. The data showed that agricultural sites had atrazine

concentrations ranging from <0.01 to 24.45 µg/L over the 6-month sampling period, but atrazine levels were not measured at the University of Miami (nonagricultural) site.

In this same study, the southern toad (*B. terrestris*) was also examined and found to have an increased incidence of intersex (Bidder's organ containing ovarian tissue) in both agricultural (14% at Belle Glade and 22% at Fisheater Creek) and nonagricultural sites (33% at Archibald Biological Station). The authors speculated that the presence of a Bidder's organ may have rendered the animals more sensitive to developmental effects. Bidder's organ is characterized as a nonfunctional, rudimentary ovary; however, no information is available on whether the organ has an endocrine function at any time during the development of the animals (Duellman and Trueb 1994; Petrini and Zaccanti 1998).

This study was useful in identifying the incidence of hermaphroditism in field-collected toads. As with the previous studies, toads with testes also appeared to have ovarian tissue, but unlike previous studies the ovarian tissue was associated with the Bidder's organ rather than the testes. While toads collected in agricultural sites may have been exposed to atrazine during development, it is unknown whether atrazine was present at the nonagricultural sites. Based on the study design, toads were also exposed to other agrochemicals which further makes it difficult to establish causality. Finally, underlying environmental conditions relevant to development of toads, *e.g.*, water quality characteristics, were not reported, which further limits an interpretation of the findings. Based on these data, it is difficult to conclude that atrazine exposure was associated with an increased incidence of intersex. Also, the study does not provide insights on the ecological relevance of the data. If toads depend on coloration to attract mates, though, the toads from agricultural sites may have impaired ability to attract mates due to their distinctly female appearance.

Crabtree, C.; E. E. Smith; J. A. Carr. 2003. Histology of the gonads and analysis of hormone levels in the native bull frog (*Rana catesbiena*) collected from agricultural areas in southern Iowa: pilot project. The Institute of Environmental and Human Health, Texas Technical University, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc. Laboratory Identification Number ECORISK Number TTU-02 (EPA MRID No. 458677-05).

This study presented the results of Phase 1 of a three-phase study in which 14 pond sites in southern Iowa (3 reference and 11 atrazine-exposed) were characterized. The overall study was designed to select sites and validate biochemical and analytical methods and organism sampling techniques to assess the effects of atrazine on kidney and gonad histology of bullfrogs (*R. catesbiena*) and other species collected from various field sites in southern Iowa. Experimental sites were located in corn and soybean-dominated agriculture areas, while reference sites were located in wooded or grassland areas. Pond sizes ranged from 0.14 to 2.94 ha with average watershed areas ranging from 2.19 to 84.02 ha. Measured atrazine concentrations in reference ponds averaged 0.06 µg/L. Mean atrazine concentrations in corn-dominated sites over the June to September time period ranged from 1.07 to 19.26 µg/L with a maximum as high as 35.07 µg/L. For soybean-dominated watersheds, the highest residues ranged from 3.19 to 3.85 µg/L. Similarly, maximum deisopropyl atrazine residue concentrations were highest in corn-dominated areas in June/July at 4.17 µg/L. Maximum desethyl atrazine (DEA) residues were highest in corn-dominated ponds at 16.55 µg/L in June/July and 16.10 µg/L in August. During the sampling period, residues of DACT remained relatively constant and the highest average residues (0.65 µg/L) were from corn-dominated ponds.

Although bullfrogs were present at all sites in sufficient numbers for collection, not all life stages were collected at every site. No significant differences were found for adult body weight or snout-vent length (SVL), but mean weight and SVL for juvenile females were significantly lower in reference sites than atrazine-exposure sites ($p=0.001$ and $p=0.0001$, respectively). Mean SVL for juvenile males was also significantly lower in reference sites than in atrazine-exposure sites ($p = 0.01$); however, mean weight of juvenile males was not statistically different between sites. Gonadosomatic index (GSI = weight of gonad ÷ body weight) was not significantly different between sites for either adults or juveniles. No gross gonadal abnormalities were

observed based on visual examination (gross morphology), and the incidence of external abnormalities was less than 1% of the total frogs collected.

This study provides information showing that bullfrogs did not exhibit a high incidence of hermaphroditism when exposed to atrazine under field conditions. In this study, the number of water samples may not provide sufficient characterization of the exposure potential to atrazine, particularly in reference sites. While an effort was made to characterize a limited number of herbicides, other pesticides were not measured. Based on the preliminary results, none of the indices measured (weight, length, GSI or the incidence of gross gonadal deformities) in the bullfrog indicated that variable exposure levels to atrazine and other triazine degradates adversely affected this species. While the bullfrog was clearly present, interpreting the field observations was difficult because information on the bullfrog relative to some of the indices of interest (*i.e.*, steroid hormone levels, aromatase levels, background incidence and types of gonadal abnormalities) were not available.

CHAPTER 3

STUDY STRENGTHS AND LIMITATIONS

Laboratory Studies

The laboratory studies reviewed by the Agency were useful in identifying measurement endpoints that may represent potential hazards to amphibians. They also provided valuable insights for designing future studies that further examine these measurement endpoints. Although laboratory studies are intended to provide an opportunity to control potential sources of variability that could affect the endpoints of interest, none of the experiments reviewed by the Agency fully accounted for environmental conditions and husbandry factors capable of influencing endpoints the studies were attempting to measure. Many open literature studies did not use standardized protocols and were therefore difficult to evaluate in the absence of confirmatory investigations. In instances where laboratory experiments used protocols and standard operating procedures that were in existence prior to initiating the studies, the observed variability associated with the various measurement endpoints suggested that study designs did not adequately account for variability, *i.e.*, sample sizes were insufficient to detect treatment effects due to large measured variances. Quality control assurance data indicated that many of the studies were subject to poor water quality. Low dissolved oxygen levels and elevated ammonia nitrogen and nitrite concentrations indicate that these factors alone could have negatively impacted growth and development of the test organisms.

In the reviewed studies, loading rates ranged as high as 30 to 65 tadpoles per liter with incomplete and infrequent exposure solution changes. These conditions could create unfavorable environmental conditions for optimum growth and development, and in fact, reported growth and development of unexposed organisms were inconsistent with patterns cited in the open literature (Nieuwkoop and Faber 1994). Laboratory manuals on the use of *X. laevis* recommend that stocking rates not exceed 1 tadpole/L and that complete water changes be made at regular intervals (Sive *et al.* 1998). Many of the laboratory studies reviewed exchanged only 50% of the

exposure solution every 72 hrs. Additionally, although laboratory studies offer the best opportunity to control exposure conditions, in several of the laboratory studies, either the exposure conditions were poorly characterized or atrazine was detected in control tanks. In some cases, the level of atrazine detected was comparable to those in atrazine-treated groups.

Feeding rates and the quality of food used were not described for many of the studies. In several studies, the authors indicated that feed analysis was "inconclusive;" however, no data were provided to independently interpret the analyses. Because the organisms appeared to be in relatively poor condition in many of the laboratory studies, it appears that feeding regimes were not adequately considered.

Generally, the plasma hormone concentrations and gonad/brain aromatase activity assays were characterized by extremely high levels of variability. Coefficients of variation ranged as high as 550% and indicated that the means to detect a treatment effect was problematic. In some cases, hormone assays could not distinguish males from females. The variability in these measurement endpoints may have been related to handling stress and protracted sampling periods. In some cases sampling extended over several hours to many months and may have resulted in a comparison of animals that were at different stages of their sexual maturation cycles. Additionally, *X. laevis* is an opportunistic breeder, and it is unclear what environmental factors initiate breeding responses. Placing males and females in close proximity to one another during collection could potentially impact plasma sex hormone levels.

Although most of the studies employed positive controls using dihydrotestosterone and 17-β estradiol, typically there was a lack of responsiveness to these hormones, implying that hormone concentrations were not sufficiently high, or that the test animals were not responsive to the chemicals, or that there were technical problems with the analytical methods used to measure sex steroid. According to the open literature, *X. laevis* treated with estradiol 72-hrs post-hatch through metamorphosis should produce 100% females (Hayes 1998); however, in the current battery of studies, estradiol treatment only yielded a maximum of 67% females and in some studies did not have any effect on sex ratios. Furthermore, measured estradiol concentrations in some of the studies were considerably lower than nominal. While it is possible that some species were not responsive to hormone treatments, it is uncertain why these species would be selected as

appropriate measures of estrogenic/androgenic effects. The current studies suggested that poor water quality, overcrowding, and insufficient hormone concentrations may have contributed to the lack of responsiveness for many of the animals to respond to estradiol and dihydrotestosterone.

The poor condition of the study animals was a recurrent theme in the majority of the laboratory study reviews. With mortality rates as high as 75% across all treatments in one study and disease in another, the viability of the test organisms was clearly in question. The protracted length of time required for the frogs to undergo metamorphosis (80+ days as opposed to the more typical 58-day period for *X. laevis*) and the tendency for animals to lose weight with increasing length of time to metamorphosis underscored EPA's concerns that study conditions were not conducive to optimal growth. The decreased condition and growth could have impacted the developmental status of the animals as evidenced by the number of frogs that did not complete metamorphosis.

Field Studies

As mentioned previously, field studies can help evaluate the relevancy and significance of toxicological effects observed in laboratory-based investigations. Unlike laboratory studies, however, the more natural conditions of the field can add variability to the data. Consequently, considerable effort is required to assure that natural variability across field study sites is accounted for and that sites do not have characteristics capable of interfering with the interpretation of the data. Most of the studies reviewed, however, did not provide sufficient information to characterize study sites or provide sufficient rationale for site selection and variability. Atrazine exposure in reference/control sites, with likely exposure to a much wider array of chemicals, reduced if not eliminated the option of conducting hypothesis testing and led some investigators to consider a regression-based approach, even though the study design was not originally intended to support such an analysis. In addition, the evaluation of potential confounding effects of non-chemical stressors, *e.g.*, habitat condition, prey availability, nutrient loading, were not described or evaluated.

While the difficulty in selecting field sites that have similar morphological characteristics is well recognized and appreciated, many of the study sites had widely divergent conditions. Although atrazine is frequently detected in monitoring studies, the potential for exposure of field study reference sites was not adequately considered in the majority of studies. Quantifiable levels of atrazine and/or its degradates were detected in many of the studies. Although some of the study authors conducted pilot studies to establish atrazine levels in experimental and reference sites, follow-up monitoring suggests that the initial surveys were misleading , *i.e*., atrazine concentrations in reference sites were considerably higher than initial sampling indicated. Additionally, other triazine herbicides (*e.g*., simazine, terbuthylazine) were detected in reference sites at comparable if not higher levels than at atrazine-exposed sites. Although other pesticides were admittedly used in the vicinity of many study locations, none of the pesticide concentrations was characterized.

Ideally, field studies should be designed based on the variability associated with the measurement endpoints, and sample sizes should reflect the number of test animals required to identify a specified difference within a given level of certainty. Potential sources of variability should also be identified and controlled to the extent possible. The current studies did not appear to be designed based on the variability associated within the range of measurement endpoints. In some cases, animals were collected over extended periods of time (up to 6 months) which could increase variability due to differences in developmental stage and reproductive status of the organisms at time of collection.

CHAPTER 4

UNCERTAINTIES IN ASSESSING POTENTIAL ATRAZINE EFFECTS

At face value, the weight-of-evidence might suggest that atrazine did not produce any consistent effects across the range of frog species tested and that the demasculinizing (decreased laryngeal dilator muscle area) effects previously reported by Hayes *et al.* (2002*a*, *b*, *c*) could not be replicated. While Tavera-Mendoza *et al.* (2001*a*, *b*) reported that exposure to higher atrazine concentrations (21 µg/L) significantly increased the incidence of gonadal abnormalities in *X. laevis*, others reported that exposure to similar concentrations had no effect in *X. laevis*. The feminizing effect (intersex/hermaphroditism) of atrazine could only be demonstrated statistically in one study (Goleman and Carr 2003), but at exposure concentrations of 25 µg/L as opposed to the 0.1 µg/L effect level reported by Hayes *et al.* (2002*a*). Additionally, the studies did not demonstrate whether gonadal and/or brain aromatase activity was induced by atrazine treatment or whether plasma testosterone/estrogen levels were affected. Although the Florida cane toad study (Goleman and Carr 2003) indicated both demasculinizing effects (genetic males with female coloration) and feminizing effects (oogenesis in male Bidder's organ), there were insufficient data to clearly link atrazine exposure to this phenomena. However, as previously discussed, each of the studies has deficiencies and uncertainties that limit their usefulness in differentiating treatment effects.

One of the issues that has been raised regarding the potential effects of atrazine on amphibian development has been the enhanced response at lower doses (*i.e.*, in the range of 0.1 to 0.3 µg/L). According to Hayes *et al*.(2002*a*), *X. laevis* and *R. pipiens* exhibited hermaphroditism at 0.1 µg/L in both laboratory and field studies, and responses at higher doses were either similar to or less than those observed at this level. Unfortunately, most of these studies only evaluated atrazine effects at concentrations ranging from 1 to 25 µg/L, and background levels of atrazine were as high as 0.1 µg/L. As a result, these studies could not be used to differentiate a "low-dose" effect since controls were often at levels at which effects have been observed in other studies.

Although these studies do not serve to reject or confirm studies claiming to demonstrate that atrazine exposure results in demasculinizing and/or feminizing effects in frogs, they were useful in identifying measurement endpoint responses that may represent potential hazards to amphibians. They also provide important information on how to design laboratory and field experiments to assess these potential effects. As previously discussed, the ability of the current battery of tests to discriminate treatment effects appears limited given the variability surrounding each of the measurement endpoints. When variability is coupled with confounding effects, such as impaired development, disease, and atrazine contamination in control/reference groups, it is difficult to draw any definitive conclusions about the test results. However, these observations provide important insights in designing studies that can minimize the introduction of variability, which is addressed in the next section of the paper (Chapter 5, Reducing Uncertainties).

Ecological Relevancy of Endpoint

In addition to the above uncertainties surrounding these amphibians studies, the ecological relevancy of the measurement endpoints used in the studies is uncertain and needs to be examined further. Even if atrazine did result in gonadal abnormalities, such as ovarian and testicular tissue in the same individual or testicular oogenesis, there are no data currently available to suggest that reproduction is impaired. Additionally, if laryngeal muscle growth were impaired by chemical exposure, are males less able to attract females by attenuated mating calls and is reproduction subsequently impaired? At present there are no data to relate the above mentioned measurement endpoints to more traditional chronic assessment endpoints of survival, growth and reproductive impairment that could impact animal populations. In Hayes *et al.* (2002c), there apparently was little difficulty in collecting specimens, even though 92% of the northern leopard frogs sampled at one site were hermaphrodites. It is uncertain, therefore, whether gonadal effects, such as hermaphroditism, significantly impaired population levels. According to the literature (Gray *et al.* 1996; DePrado *et al.* 2000), resistance to atrazine has developed in many species of weeds and nontarget plants. Hayes *et al.* (2002*a*; *c*) has suggested that chemical exposure may result in

resistance to the proposed feminizing effects of atrazine by delayed metamorphosis. However, there are no data currently available to assess a possible compensatory mechanism.

Dose-Response Relationships

Hayes *et al.* (2002*a*, *c*) has suggested that developmental effects resulting from atrazine exposure are nonmonotonic and do not follow typical dose-response curves where higher concentrations elicit greater effects. Rather, the response of both clawed frogs and leopard frogs has been either a trigger followed by a consistently similar response or an enhanced response at low concentrations followed by diminished, although significant, effects at higher concentrations (low-dose effect). Although accentuated responses at lower doses have been associated with herbicides in an effect termed hormesis (Calabrese and Baldwin 2001), the relevancy of this effect to risk assessment has not been established. Because most of the current studies did not test below 1 µg/L and many of the reference sites were contaminated with atrazine at levels where other studies have claimed to demonstrate accentuated responses, there is not sufficient information available to ascertain the reproducibility of a potential low-dose effect.

Mechanistic Plausibility of Atrazine Effects

Successful extrapolation of the effects of a chemical among different species is largely dependent on the degree to which biological processes are conserved. Conservation in biological processes are typically highest in basic cellular, early ontogenetic, and endocrine mechanisms. For example, the mechanisms associated with the synthesis, metabolism, and receptor activity among vertebrates for estrogen, androgen, and thyroxine are considered to be generally highly conserved, albeit some differences exist. The "downstream" effects of these hormones which occur subsequent to receptor binding, on the other hand, tend to be divergent. Therefore, the probability of successful inter-species extrapolation is highest when analyzing the "upstream"

processes, that is, those that are typically associated with homeostatic mechanisms and receptor binding.

In the case of atrazine, the premise has been that aromatase induction results in elevated estrogen levels which leads to the apical effect, *i.e.*, ovotestes and reduced secondary sex characteristics in males (Hayes *et al.* 2002*a*,*c*). Aromatase induction by atrazine, though, has not been demonstrated in any anuran in the laboratory, and attempts to replicate hermaphroditism in other studies have not been successful. While Hayes *et al.* (2002*a*) reports that adult males treated with 25 µg/L atrazine had significantly lower ($p<0.05$) testosterone levels compared to control males, aromatase activity was not measured in these animals. None of the studies reviewed demonstrated a statistically significant increase in aromatase activity following atrazine exposure. Based on the Agency's understanding of the variability associated with the aromatase assay used in these analyses, the study designs were not sufficient to detect treatment effects. Consequently, at this time there do not appear to be data available that would directly support a hypothesis that aromatase induction in amphibians occurs as a result of atrazine exposure. If the inability to demonstrate aromatase induction is related to problems in replicating study-specific conditions, then it is important to consider whether those conditions are environmentally relevant and whether this introduction of variability represents an essential design component for future studies to more fully evaluate the potential role of aromatase induction. If an endpoint were to be identified, then the normal pattern would need to be described in both field and laboratory settings to discriminate actual deviations.

Laboratory to Field Extrapolation

A major challenge to interpreting the ecological significance and relevancy of effects observed in the laboratory is, in part, a function of the difficulty in extrapolating responses observed under relatively greater controlled and monitored treatment exposures and environmental conditions to the more variable and uncontrolled nature of exposures and conditions in the field. For example, typical laboratory conditions for *X. laevis* utilize constant water temperatures (20-25^{o}C) and constant photoperiod (12 hrs light:12 hrs dark). These conditions maintain *X. laevis* adults in a continual reproductive status in which the animals can be induced to spawn. However, in the field, organisms are subjected to fluctuating (diurnal and seasonal) temperatures and to continuing changes in photoperiod, according to the season. These two environmental factors, temperature and photoperiod, are known to be key determinants of reproductive status in most aquatic organisms. As a result of these differences in environmental factors, the physiological status of organisms in the field may differ significantly from those in the laboratory. Finally, the studies conducted so far have not taken into account the developmental biology of *X. laevis*, nor have they considered fluctuations in atrazine exposure. For example, the extended sampling periods could result in comparing organisms at different stages of their annual reproductive cycles and months after atrazine application.

Application of Available Studies to Assess Potential Atrazine Effects

Based on the currently available data, the Agency has determined that there is sufficient certainty and consistency across several studies (Tavera-Mendoza *et al.* 2001*a,b*; Hayes *et al.* 2002*a,b,c*; Goleman and Carr 2003; and Sepulveda and Gross 2003) to establish the plausibility of a hypothesis that atrazine could affect amphibian development. However, the uncertainties

described previously preclude establishing a definitive characterization of atrazine's effects on amphibian development. Further, based on the available information, conclusions regarding the magnitude and likelihood of any potential adverse developmental effects span an approximately 250-fold range of atrazine exposure, due to the existing uncertainties. If the Agency's risk management decision requires a greater degree of certainty in the ecological risk assessment for atrazine than possible from currently available data, then additional data would be necessary to evaluate the potential causal relationships between atrazine exposure and gonadal development in amphibians and if so, the nature of the dose-response relationship. To the extent that any mode of action for any observed effect(s) can be proposed and supported, such information would strengthen the plausibility of the relationship. Although field studies provide useful insights on "real-world" responses and reflect what may actually occur in a natural setting, they may be subject to a wide range of environmental effects that can influence a study's measurement endpoints beyond treatment effects. The current studies show the extent to which field experiments can introduce variability and make it difficult to identify atrazine-specific effects. As described in Chapter 5 (Reducing the Uncertainties), EPA recommends that reliable laboratory experiments be conducted before field studies are undertaken. Additionally, EPA welcomes discussions with the SAP on potential future study protocols to help establish a perspective for both the Agency and the scientific community to consider in reviewing experimental design options before initiating any experiments intended to address the uncertainties described in the current evaluation.

CHAPTER 5

REDUCING THE UNCERTAINTIES

As discussed in Chapter 4 , there are numerous uncertainties associated with the studies reviewed for this paper. While there is sufficient information to establish a plausible hypothesis concerning potential atrazine effects, the uncertainties associated with these studies are significant, making it difficult to address the core risk question as to whether or not atrazine can adversely impact anuran development and reproduction. Current studies show that field experiments may introduce considerable variability and make it difficult to identify atrazine-specific effects. Therefore, before additional resources are devoted to field studies, laboratory studies should be designed and conducted regarding the working hypothesis that atrazine exposure causes gonadal developmental effects in amphibians. Once this effect can be established, with a corresponding dose-response relationship, resources should be devoted to examining induction in aromatase activity, which elevates circulating estrogen concentrations and in turn results in the feminization of male anurans. EPA recommends that study protocol characteristics of higher tier studies be reviewed in some detail by the SAP in a future consultation, assuming a review of the current Agency analysis is consistent with the Panel's evaluation.

The tiered approach for conducting these studies, which is partitioned into five distinct phases, follows a typical reductionist approach (**Figure 1**). Briefly, the objective of the first phase is to conduct studies that determine whether or not atrazine exposure causes changes in apical effects (*e.g.*, hermaphroditism) related to gonadal development and reproduction. A secondary objective of this phase is to provide information on the repeatability of previous observations, develop a sound dose-response relationship, and determine the developmental sensitivity of the

test species, *X. laevis*. If apical effects are observed in response to atrazine exposure, then three additional phases are proposed that address the mode and mechanism of atrazine action along the lines of the working hypothesis. Before proceeding through the proposed study phases, a risk management decision would be required to determine if further reduction in uncertainty is needed.

In the second phase, sex steroid (*i.e.*, testosterone and estrodiol) measurements would be conducted, and in the third and fourth phases aromatase activity would be measured. These mechanistic studies would be conducted for three reasons. First, understanding the mode of action is essential for extrapolating between species. Because it is impossible to test all species of concern for apical effects, this overall approach uses *X. laevis* as a surrogate species where dose-response, developmental sensitivity, and mechanism of action data are used as the basis to investigate the effects of atrazine on native species. This approach also allows for studies with native anurans to be focused and efficient and permits one to test the hypothesis that native species respond similarly to atrazine as *X. laevis.*

Second, mechanistic information is important in that it reduces the uncertainties associated with developing a causal relationship between apical effects and atrazine exposure by demonstrating a plausible, mechanistic basis for any such effects. In this context, such data also assist with the interpretation of dose-response relationships and appropriate dose metrics. Finally, mechanistic studies set the stage for the development of potential bioindicators of response that can be applied if future field work is needed. The lack of such information is a critical factor that limits the utility of the field studies conducted thus far.

Phase 5 of this proposal attempts to develop data to establish whether or not the effects of atrazine on gonadal development, if established, are ecologically relevant. This phase would require some novel approaches for evaluating fertility of males after atrazine exposure in order to develop data suitable to an analysis with population models.

There are no universally accepted standardized methods for the studies outlined above. Therefore, the methods proposed in the following sections represent an *ad hoc* approach, using information and experience of several different laboratories. Although this proposal defines a series of studies to develop a comprehensive understanding of atrazine's potential effects at the organismal and sub-organismal levels, this approach does not need to be fully executed as currently outlined. If the studies proceed from the initial phase to other phases, then there is an opportunity to review the outcomes and revise future study objectives accordingly and to evaluate the utility of conducting the next phase relative to the potential improvement of an eventual risk assessment. The advantage to this approach is that it minimizes the generation of unnecessary data, thus minimizing costs and delays in completing the risk assessment. This flexibility, however, requires iterative review and communication between the risk assessors and risk managers to ensure that the Agency's data needs are met.

Phase 1. Test for Apical Gonadal Effects

The ability of only one laboratory to produce ovotestes at low atrazine concentrations is problematic because this is the endpoint on which much of the concern hinges. Therefore, additional studies on the effects of atrazine on gonadal differentiation should be conducted. Repeatability is the hallmark of sound science and must be considered as an important step in determining the relevancy of previously conducted studies. Positive results, besides supporting

the conclusions of previous studies, would provide the rationale for conducting further studies which attempt to elucidate the pathway of potential atrazine effects. Negative results, on the other hand, that do not support the previous studies would eliminate the need to proceed to additional phases of investigation.

Species

The species used for this phase should be both amenable to laboratory experimentation and sensitive to the effects in question. *Xenopus laevis* and *Rana pipiens* meet both of these requirements. *X. laevis*, however, should be used as the primary species because of its ease of culture, shorter developmental time, and the ability to conduct studies throughout the year. *Rana pipiens*, while perhaps more environmentally relevant to North America, should only be used in focused studies for additional confirmation of results obtained using *X. laevis*.

Stage Sensitivity

These studies should first be initiated with free swimming larvae (96-hr post-fertilization; NF Stage 46) to make a direct comparison to the previously published studies and to include the developmental stages known to be sensitive to feminization by exogenous estrogens (Villalpando and Merchant-Larios 1990; **Figure 2**). Exposures should continue through metamorphosis (NF Stage 66), at which time the study can be terminated. If atrazine is shown to induce gonadal effects in larvae, then additional studies should be conducted at the effective atrazine concentrations. These studies should initiate exposure with different developmental stages (*e.g.* NF stages 52 and 58) to determine if there are stage sensitivity differences and whether those differences are consistent with previous studies (Villalpando and Merchant-Larios 1990; **Figure**

2). By establishing stage sensitivity, further experimentation in subsequent phases could be more focused.

Test Conditions

The tests should be conducted using flow-through conditions which favor growth, survival, and development. Static or static renewal methods should not be used. The type of water is not specified in this method because there is no known optimal water type. Instead, the type of water used must be established to promote normal survival, growth, and development under the conditions of the proposed studies. Using a flow-through system, there is no need to aerate the exposure water. Feeding should be done on a daily basis using a food type and quantity that also has been demonstrated to promote normal survival, growth, and development.

Biological loading rates are a major concern of the previously conducted studies. Loading rate is known to be an important factor in maintaining adequate water quality which is essential for valid aquatic toxicity testing. Excessive loading diminishes water quality through metabolic activity and respiration and can result in inefficacious exposures. Standards have been established by the American Society for Testing and Materials (ASTM 1998) for acceptable loading rates of fishes, amphibians, and invertebrates in both static (or static renewal) and flow-through conditions. ASTM (1998) standards for loading in tests conducted using static renewal conditions at 22^{o} C are 0.5 g/L of test solution. All of the studies reviewed in this report exceed this recommendation and suggest that the studies failed to maintain adequate conditions for a valid bioassay (loading rates were from 1.1 to 24.4 g/L; see **Table 2**).

Table 2. Summary of loading rates used in the various laboratory studies on the effects of atrazine on amphibian gonadal development.

	Exposure Type	Species	Number of Tadpoles per Replicate	Maximum g/organism	Total g	Liters of solution	g/L
Hayes *et al.* 2002*a*	static renewal	*X. laevis*	30	1.5[1]	45	4	11.3
Goleman and Carr 2003	static renewal	*X. laevis*	60	1.5[1]	90	4	22.5
Hayes *et al* 2002*c*	static renewal	*R. pipiens*	30	2.5[2]	75	4	18.8
Hecker *et al.* 2003.	static	*R. clamitans*	30[3]	2[4]	60	4	15.0
	renewal		20[5]	2[4]	40	16	2.5
Du Preez *et al.* 2003.	static	*X. laevis*	800	1.5[1]	1,200	1,100	1.1
Goleman and Carr 2003.	static renewal	*X. laevis*	65	1.5[1]	97.5	4	24.4
Tavera-Mendoza *et al.* 2001*a*; 2001*b*.	static	*X. laevis*	16	1.5[1]	24	15	1.6
Hecker *et al.* 2003	static renewal	*X. laevis*	30	1.5[1]	45	4	11.3

[1] not reported; estimated based on data provided in **Figure 3.**

[2] not reported; estimated based on Ankley *et al* (1998).

[3] initial number, but high mortality confounds analysis

[4] not reported; estimated based on metamorph weights

[5] not reported; estimated based on approx. 30% mortality in controls at transfer to larger tanks on day 67

The ASTM-recommended loading rates for studies conducted using flow-through conditions is 1 g/L for every liter dispensed in 24 hrs. Current studies conducted with *X. laevis* at the EPA, Office of Research and Development's Mid-Continent Ecology Division (MED) employ flow-through conditions that were designed to meet the ASTM standard. Typically, the flow

rates in each exposure tank are 0.025 L/min, which is equivalent to 36 L/day. Biological loading is usually 20 organisms, which reach about 1.8 g maximum body weight (**Figure 3**), resulting in an approximate loading rate of 1 g/L for each liter dispensed in 24 h. Under these conditions, minimal mortality has been observed, and metamorphosis is typically completed in about 55 days (personal communication: J. Tietge, U.S. EPA, Mid-Continent Ecology Division, 2003).

Water concentrations of atrazine and its major degradates, *i.e.*, diaminochlorotriazine (DACT), desethylated atrazine (DEA), and desisopropyl atrazine (DIA), should be measured in duplicate at specified frequencies using an appropriate analytical method to cover the concentration range of the parent compound used in the test. Water chemistries (*i.e.*, dissolved oxygen, pH, ammonia) should be measured periodically throughout the study and reported.

Dose-Response

The studies to date provide inconsistent information as to the nature of a dose-response relationship for potential atrazine effects. Further studies suggest that lower concentrations of atrazine are more effective than higher concentrations in eliciting gonadal effects although no biological basis has been demonstrated for this response. However, without knowing the biological basis, the shape of a dose-response curve can only be empirically established by testing multiple concentrations in the range of interest. The highest test concentration should be at or above 25 µg/L, while the lowest should be below 0.1 µg/L. If the results of the initial study using this concentration range demonstrate non-monotonic dose-response patterns, then subsequent tests that focus on a specific concentration range may be indicated. If atypical dose-response patterns are observed, *i.e.*, inverted U shape dose-response, then additional work may be necessary to develop a biological basis for such a response. This may require dosimetry

measurements to ensure that the organismal dose, *i.e.*, whole organism residue, is correlated and dependent upon the applied dose, *i.e.*, exposure water concentration. Deviations from such a relationship could indicate that other mechanisms, *e.g.*, metabolism, active uptake, active elimination, are at work to alter the expected steady-state relationship, thereby potentially altering the shape of the dose-response curve.

Positive Controls

Many of the atrazine studies have used "positive" controls. Typically they have used both an estrogen and an androgen. The use of an estrogen is desirable because it demonstrates the sensitivity of the organisms to a potential estrogen effect that is hypothesized to be in the pathway impacted by atrazine. Estrogen applied concentrations should be measured at specified intervals to assure adequate delivery to organisms. The use of an androgen as a "positive" control is not necessary because there is no plausible relationship, based on the working hypothesis, between androgen responsiveness and the effects of concern.

Sampling

Sampling of all organisms under study is essential to avoid potential bias related to differences in developmental rate. Sampling should occur at the same developmental stage to make sure that the inter-organism comparisons are valid. For *X. laevis*, sampling when each organism reaches NF 66 is appropriate. Replication, loading, and final sampling should be determined using statistical procedures to ensure that the hypothesis of the study can be tested appropriately.

Endpoints

The primary endpoints of interest are those indicative of abnormal sexual differentiation. These include: gross analysis of chemically prepared (fixed) gonads to determine the morphological condition of the gonads, histological analysis of gonads from each organism to determine whether effects such as testicular oocytes and reduced spermatogonial cell nests occur in males, and alterations in the numbers of primary and secondary oogonia occur in females. Additionally, determination of male:female sex ratios and other observations on secondary sexual characteristics should be made. Evaluation of laryngeal musculature is not necessary, as the gonads appear to be more sensitive to the effects of atrazine exposure. In addition, the typical endpoints of daily survival, growth (as determined by wet weight at the termination of the study), and development (as indicated by time to complete metamorphosis) should be evaluated.

Quality Indicators

Because there are currently no standardized methods available for reproductive and developmental assays with anurans, in general, nor with *X. laevis* specifically, the following guidelines are proposed as quality indicators of a valid study. Of utmost importance, the study should attempt to conform to the ASTM recommended standards for biological loading rates of 1.0 g/L for flow-through studies. These loading rates should be determined using the maximal mass of the organisms, not the initial or final masses, as considerable weight loss occurs (up to 50%) during metamorphic climax. Minor deviations can be tolerated, but the ASTM guidance should be strongly considered in test design and conduct.

Survival is obviously an important indicator of study quality. And, while there is no bright line between acceptable and unacceptable survival rates, survival of 90% or more of the organisms indicates a quality study, particularly in the controls. Although the level of

unacceptable mortality is not defined, excessive mortality serves to bias the results of the study by limiting analysis to the survivors.

Like survival, there are no set criteria for acceptable growth and development. However, taken in aggregate, growth and development should be as optimized as practical. Maximal larval weights of *X. laevis* should be about 1.5 to 1.8 g, with weights of NF 66 organisms approximately half of maximal weights (**Figure 3**). Development in controls should proceed without great deviations from those determined by Nieuwkoop and Faber (1994), with metamorphic completion in approximately 7 to 9 weeks. Conditions that delay development of unexposed organisms beyond 10 weeks suggest problems with food or water quality and introduce uncertainties regarding the effects of overall delayed development on system specific development. Although there are little or no known data regarding delayed metamorphosis on organ-specific development, there is a large body of literature on the effects of accelerated development demonstrating that accelerated metamorphosis, through exposure to thyroid hormone, results in abnormal system development (Huang *et al.* 2001). Clearly, development is a coordinated event with temporal constraints.

Besides the biological indicators, there are several standard exposure-related measurements that are indicators of a quality study. The most common are dissolved oxygen, ammonia, and pH. ASTM guidelines suggest that dissolved oxygen should be maintained between 60 and 100% of saturation (ASTM 1998). These guidelines recommend that pH be within an acceptable range for the test organism, which in the case of *X. laevis* is suggested to be within 6.5 and 9.0 for earlier developmental stages used in the FETAX assay (ASTM 1998). While no specific guidance is provided for ammonia concentrations, two studies have evaluated

the toxicity of ammonia to *X. laevis* larvae and found that concentrations of 25-50 ppm are lethal and that concentrations of about 15 ppm significantly reduced growth (Tietge *et al*. 2000; Schuytema and Nebeker 1999) in short- term studies. These studies suggest that total ammonia concentrations should be well below 10 ppm because total ammonia (recognizing that the toxic form, unionized ammonia) is dependent on the pH of the test media.

Analysis, Interpretation, and Iteration

If the study or studies indicate that atrazine affects gonadal differentiation, then the experimentation could move to the next phase or phases which deal with questions regarding the mechanism of action and reproductive fitness. Although these mechanistic and higher order questions are presented sequentially, they could be evaluated simultaneously.

Negative results, however, present a much more difficult problem. At issue is whether or not a study design is, indeed, a valid attempt to repeat previous studies using the same conditions. Strictly speaking, the conditions of any of the atrazine studies cannot be repeated because it would be highly improbable to have organisms of the same genetic makeup, which could be a contributing factor to the outcome. The conditions recommended here do not attempt to actually replicate the conditions of any of the atrazine studies conducted thus far. Those conditions appear to have been inadequate to promote normal survival, growth, and development. The underlying assumption is that a biological phenomenon, if significant, is pervasive under a broad range of conditions that are favorable to the viability of the organism being tested. If effects are only observed in the laboratory due to specific test conditions that are not likely to be found in the field, then those effects may not be relevant. If, however, the studies are negative, after

reasonable attempts are made to use well-established aquatic toxicity methods in a systematic approach, then there is no plausible rationale to proceed with the next phases of the investigation.

Phase 2: Sex Steroid Measurements

If positive effects are observed in Phase 1, then it is logical to hypothesize that estrogen levels are increased and result in feminization. At this point, with the stage and dose dependency of the effects documented, it should be possible to construct a refined exposure protocol which maximizes a presumptive increase in estrogen concentrations in organisms at specific developmental stages. Kloas (2002) has demonstrated the ability to measure estrogen in organisms at early developmental stages, including those that presumably would be present in this hypothetical study.

If estrogen levels are determined to be elevated, then it is reasonable to propose moving into Phase 3, which proposes to measure aromatase activity. If estrogen levels are not elevated, then an alternative path should be followed, which evaluates other mechanisms associated with gonadal differentiation. Since the alternative path is not part of the working hypothesis, no further discussion of it will be presented here.

Phase 3: Aromatase Activity Measurements

The test for increased aromatase activity as a result of atrazine exposure at the appropriate developmental stages assumes that estrogen levels have already been elevated by atrazine. The question is whether or not elevated estrogen levels result from enhanced aromatase activity. Aromatase, the enzyme responsible for conversion of testosterone to estradiol, is expressed in several tissues of *X. laevis*. The developmental expression of this enzyme has been determined and detectable expression of aromatase mRNA in the gonad occurs at NF Stage 51. A small

transient rise of expression occurs at Stage 53, followed by a transient depression at Stage 54, and a large rise of expression after Stage 55 (Miyashita *et al.* 2000; **Figure 3**).

Because exposure to exogenous estrogen results in feminization of the male phenotype, it is reasonable to hypothesize that enhanced aromatase activity could lead to abnormally elevated endogenous estrogens that can also affect feminization of males. Under normal developmental circumstances, the activity of aromatase is either absent or very low during the developmental stages that are most sensitive to feminization by estrogens, suggesting that this phenomenon does not occur naturally (**Figure 3**). In order for aromatase activity to effect feminization of the testes, the activity of this enzyme must be sufficiently elevated during the developmental period sensitive to estrogen-induced feminization. This requires that atrazine cause aromatase expression to occur prematurely and at high enough levels to produce sufficiently high endogenous estrogen levels to affect feminization.

If, on the other hand, atrazine does not increase aromatase activity, then consideration should be given to studying its effect on other factors that are involved in the homeostasis of estrogen. A likely research avenue would include studies on hypothalamic and pituitary factors, which have already been shown to be affected by atrazine in rodents (Cooper *et al.* 2000).

Phase 4: Aromatase Inhibitor Study

Assuming that aromatase activity is elevated by atrazine, a logical confirmatory study would be to expose organisms to atrazine in combination with an aromatase inhibitor. This study could be performed at all of the preceding levels and incorporate apical endpoints as well as sex steroid and aromatase measurements.

Phase 5: Ecological Relevancy Study

Quantifying the likelihood of population-level responses due to the potential effects of atrazine exposure to anurans is challenging. The hypothesis is that atrazine exposure adversely affects reproductive fitness of a specific anuran species to the extent that population levels decline in response. To test this hypothesis, an anuran species that permits the analysis of reproduction is required. Currently, there are no known laboratory methods to evaluate this endpoint directly with native species. And, even though reproduction in *X. laevis* appears to be a useful surrogate model to identify the effects of atrazine on development of the reproduction system, it probably cannot be used as a surrogate for determining ecological effects on native anuran species.

The essential elements of determining reproductive fitness can be readily identified, but collecting data to confidently assess them is relatively difficult. Because the primary effect of concern is related to males, the primary endpoint of interest is the ability of males to attract mates and fertilize eggs during mating (amplexus). Fecundity, per se, as an indication of female reproductive capacity is of little value, unless further experimental data support the results reported by Tavera-Mendoza *et al.* (2001*b*), which suggest that adverse gonadal effects occur in females as well.

The essential elements affecting the male's ability to effect egg fertilization can be broken down into three main components: gamete function, gamete release, and reproductive behavior. The first, gamete function, can be assessed using *in vitro* fertilization methods. Briefly, this method requires that previously exposed males are either maintained under temperature and lighting conditions that induce reproductive readiness or are induced using pituitary extracts or gonadotropins. Testes would be removed and macerated in order to release the spermatocytes,

which would be applied to a fixed number of previously collected oocytes. The efficacy of fertilization could then be analyzed by determining the prevalence of developing embryos. This type of methodology is routinely used in *X. laevis* and *X. tropicalis* and could be adapted for use in native species that are amenable to the procedure. In the case of most native anurans, this would require exposing the organisms during the period of atrazine sensitivity, followed by 1.5 to 2 years of holding to allow for sexual maturation to occur. Once again, although testing the ranid species could eventually be necessary, it may be advisable to first use *X. tropicalis*, which reaches sexual maturity in about six months, to determine if there is a fertility hazard. If a significant reduction in fertilization is observed, then histological analysis of male gonads of native anurans may be an important consideration in extrapolating potential effects on spermiogenesis among native and non-native species.

If *in vitro* fertility is unimpaired, then it would be advisable to determine the ability of the males to successfully release the gametes. This concern is based on the fact that the presence of ovotestes alters the structure of the gonad, which may impede gamete release. This type of study would require an *in vivo* approach using induced reproduction.

Because the reported effects on the gonads result in ovarian tissue occurring in males, it is possible that males with ovotestes have elevated circulating estrogen concentrations. This could impact the normal endocrinological control of reproductive behavior and secondary sexual characteristics related to reproduction, as suggested by the Florida cane toad study where males expressed female coloration patterns and lacked nuptual pads (Sepulveda and Gross 2003). The only viable way to test for these effects is to use natural reproductive behavior in an *in vivo* study. These are the most difficult studies to successfully conduct.

Finally, the data collected from fertility studies may need to eventually be translated into an estimate of reproductive output. The changes in reproductive output would in turn need to be expressed in probability terms associated with atrazine exposure. Finally, population models would need to be developed or applied to assimilate the reproductive output data.

CHAPTER 6

CONCLUSIONS

A significant body of information is available to evaluate the potential effects of atrazine on amphibian development. Clearly, the body of studies reflect the commitment of an international group of research teams to independently examine whether atrazine exposure results in effects on gonadal development, and these investigations provide useful information to help resolve this complex scientific and risk assessment challenge. Any future research will certainly benefit from and build upon this existing foundation.

Based on a review of available studies in the open literature along with recently submitted registrant-sponsored studies examining the effects of atrazine on gonadal and laryngeal development in frogs, the Agency has concluded that none of the studies fully account for environmental and animal husbandry factors capable of influencing endpoints that the studies were attempting to measure. Therefore, as discussed below, EPA finds the overall weight-of-evidence so uncertain that it does not support any definitive conclusions with regard to whether or not atrazine exposure adversely affects amphibian development.

The current weight-of-evidence does not show that atrazine produces consistent, reproducible effects across the range of exposure concentrations and amphibian species tested. Only one study (Hayes *et al.* 2002*a*) has demonstrated statistically significant reductions in laryngeal muscle area (demasculinization) in atrazine-exposed males. Additionally, while Hayes *et al.* (2002*a*, *b*) have reproduced "feminizing" gonadal developmental effects (hemaphroditism/ovotestes/intersex) in males at atrazine concentrations as low as 0.1 µg/L, similar gonadal effects have only been demonstrated in one other laboratory (Goleman *et al.* 2003) at 25 µg/L for *X. laevis* at roughly the same length of exposure and stage of animal

development. The only other laboratory studies demonstrating gonadal effects (Tavera-Mendoza *et al.* 2001a,b) followed a considerably shorter (48-hr) exposure to a single atrazine concentration (21 µg/L) and resulted in dissimilar endpoints, *i.e.*, reduced testicular volume and numbers of spermatogonial cell nests in males and decreased numbers of primary and secondary oogonia in females. While Hayes *et al.* (2002*b*,*c*) demonstrated feminizing effects in male leopard frogs collected in the field, the incidence of hermaphroditism varied widely relative to atrazine concentration. In another field study, Sepulveda and Gross (2003) demonstrated increased incidence of hermaphroditism/intersex in cane toads and southern toads collected in Florida, but the relationship to atrazine exposure was uncertain.

Although the weight-of-evidence does not show that atrazine produces a consistent, reproducible effect, both laboratory and field studies provide evidence that atrazine exposure may be associated with effects on gonadal development and secondary sexual characteristics. The Agency believes that the lack of reproducibility across studies, which may, in part, be due to an inconsistency in the methods used by the various research teams, and the absence of a dose-response at this point do not refute the hypothesis that atrazine exposure may result in gonadal developmental effects in amphibians. Studies conducted by Hayes *et al.* (2002*a*,*b*), Tavera-Mendoza *et al.* (2001*a*,*b*) and Goleman *et al.* (2003) suggest that atrazine exposure at various levels resulted in some degree of gonadal developmental effects and thus serve to identify a potential hazard to *X. laevis*. However, none of these studies permit a clear understanding of how potential gonadal effects vary with exposure.

While this paper evaluated the experimental designs employed in each of the studies reviewed in some detail, it is important to note that none of the open literature studies were conducted for regulatory purposes nor were specific protocols available to assist in the design of

registrant-sponsored studies. Notwithstanding the limitations on the available data, collectively, the research has provided valuable and useful insights into sources of variability that can facilitate future study designs to reduce uncertainties.

In summary, while the current research does not support a definitive conclusion regarding a quantitative dose-response relationship between atrazine exposure and effects on gonadal development, it provides sufficient information to formulate a hypothesis that atrazine exposure may affect gonadal development. If the Agency's risk management decision requires a greater degree of certainty in the ecological risk assessment for atrazine than possible from currently available data, then additional information would be necessary to evaluate the potential causal relationships between atrazine exposure and gonadal development in amphibians. Any future studies should take into account lessons learned from the currently available research. While the Agency does not discount the utility of field-based studies for examining effects under "real-world" conditions, it believes that at this time laboratory-based studies provide the best opportunity to control environmental and animal husbandry factors in order to demonstrate whether a quantitative relationship exists between atrazine exposure and effects on gonadal development.

In the next chapter (Charge to the Panel) the paper solicits input from the FIFRA Scientific Advisory Panel on the Agency's interpretation of the existing data regarding the effects of atrazine on amphibian gonadal development. Additionally, the Agency is seeking input on its proposal to further test the hypothesis that atrazine exposure results in gonadal developmental effects.

CHAPTER 7

CHARGE TO THE PANEL

The Agency has reviewed relevant studies in the scientific literature, as well as studies submitted by the registrant, to evaluate the potential for atrazine to elicit developmental effects in amphibians. The strengths and limitations of the individual studies were assessed, and the extent of concordance for the entire body of information derived from these laboratory and field studies was considered to assess the plausibility that atrazine can cause developmental effects, and if so, the nature and strength of associated dose-response relationships. These developmental effects included time to metamorphosis, growth, gonadal abnormalities, sex ratios, laryngeal dilator muscle area, plasma steroid concentrations and aromatase activity of brain and gonadal tissue.

The Agency has identified a number of uncertainties in the previous studies' attempts to demonstrate whether atrazine affects development in amphibians. To resolve these uncertainties, the Agency has outlined a conceptual model for studying atrazine action on amphibian development and has proposed an approach using focused empirical studies to test the hypotheses embedded in the conceptual model.

The Agency is seeking feedback from the SAP on a number of questions surrounding the current body of evidence regarding the potential effects of atrazine on development in amphibians and the relevancy of these potential effects to ecological risk assessment. Additionally, the Agency is requesting that the SAP review and comment on the conceptual model for potential future studies.

Questions

1) In reviewing the available laboratory and field studies, the Agency used a number of criteria to evaluate individual investigations. Criteria such as experimental design, test protocols, and quality assurance information were used to ascertain the reliability of the generated data in terms of its ability to adequately assess a hypothesis that atrazine elicits developmental effects in amphibians, and if so, the nature and strength of associated dose-response relationships.

a) Does the SAP have any comments and recommendations regarding the EPA's approach and criteria used to evaluate the studies?

b) Given the evaluation criteria employed by the Agency, please comment on EPA's overall characterization of the currently available studies.

c) Please comment on the availability, as of February 28, 2003, of additional, relevant studies in the open literature that were not addressed in the white paper.

Since February 28, 2003, is the Panel aware of any studies that would be relevant?

2) In its evaluation of existing field studies, the Agency has concluded that these investigations are of limited value. The reasons include: (1) the high variability in environmental conditions and uncertainties in the pre-existing status and condition of field-collected animals, (2) the spatial and temporal aspects of atrazine exposure (*i.e.*, spatial and temporal variability over the course of the studies and the extent to which such aspects of atrazine exposure were empirically measured or otherwise accounted for), and (3) the possible co-occurrence of additional chemical and/or non-chemical stressors.

a) To the extent that the field studies appear to indicate that atrazine may not adversely affect development, please comment on EPA's conclusion that the body of data from field studies does not provide the means to ascertain whether the lack of a relationship between atrazine exposure and developmental effects is due to the absence of a causal relationship or limitation in study methodologies.

b) To the extent that any field studies appear to indicate that atrazine may adversely affect development, please comment on EPA's conclusion that these field studies do not provide sufficient information to resolve the potential role of additional co-occurring stressors.

3) In an evaluation of the existing laboratory-based studies, the Agency concluded that there was sufficient information to establish a hypothesis that atrazine could cause adverse gonadal developmental effects. However, due to different experimental designs and variability in the nature and extent of experimental conditions (*e.g.*, level of excessive mortality, delayed development in untreated organisms, lack of response to positive controls) it was not possible to adequately assess the hypothesis that atrazine causes developmental effects. It was further concluded that the current body of information did not provide the means to characterize the nature of any associated dose-response relationships.

a) Please comment on EPA's determination that the laboratory studies provide a plausible basis for the means to establish a hypothesis concerning the potential for atrazine to cause developmental effects. Also, please comment on whether the overall body of available data is adequate to demonstrate whether or not atrazine causes developmental effects under the conditions described in these studies.

b) Please comment on EPA's conclusion that given the variability in the available dose-response data across the studies (*e.g.*, an approximately 250-fold difference in reported thresholds for observed developmental effects as well as reports of monotonic and non-monotonic dose-response curves), it is not possible to ascertain the relationship, if any, of atrazine exposure to developmental effects in amphibians.

4) Many of the available studies proposed that aromatase induction results in elevated estrogen levels that lead to feminization (ovotestes/intersex/hermaphroditism) in genetically male amphibians.

a) Please comment on EPA's conclusion that, to date, aromatase induction by atrazine has not been demonstrated in any anuran in controlled laboratory investigations.

b) The variability associated with plasma sex steroid concentrations and aromatase activities is high. Is this variability normal? Please comment on any readily apparent or available methodological improvements (*e.g.*, changes in sampling design, analytical techniques) that could efficiently address this variability in future studies.

c) Please comment on whether there are additional data, other than those summarized in the white paper, that suggest late exposure of amphibians (*i.e.*, juveniles or adults) to estrogens or estrogenic chemicals can induce ovotestes formation.

d) Please comment on whether there are additional data, other than those summarized in the white paper, that suggest alternative mechanisms that could explain the apparent feminization of genetically-male amphibians.

5) With regard to specific endpoints, the Agency does not currently have sufficient information to quantitatively relate gonadal/laryngeal effects to reproductive outcomes. A major underlying uncertainty is the ecological relevance of ovotestes occurrence to the maintenance of anuran populations.

a) Can the Panel provide sources of data on background rates of ovotestes occurrence in amphibian species and any associated considerations for interpreting this information in the context of the reviewed studies?

b) Can the Panel characterize any evidence that suggests that the presence of ovotestes in male anurans results in reproductive impairment via reductions in fertility?

c) The reduction of laryngeal muscle area suggests diminished testosterone in males. If this is found to be a valid observation and if estrogen concentrations do increase as testosterone concentrations decrease, what other endpoints (*e.g.*, secondary sexual characteristics and reproductive behavior) would likely be affected?

6) While some of the available data indicate there may be an association between atrazine exposure and developmental effects in amphibians, the Agency's evaluation of the existing body of laboratory and field studies has determined that there is not sufficient scientific evidence to indicate that atrazine consistently produces effects across the range of amphibian species examined. However, the current body of knowledge has deficiencies

and uncertainties that limit its usefulness in assessing potential developmental atrazine effects and the extent of any associated cause-effect and dose-response relationships. Consequently, the Agency has determined that there are not sufficient data to reject the hypothesis that atrazine can cause adverse developmental effects in amphibians.

a) Does the SAP concur with these conclusions? If not, what lines-of-evidence would lead to an alternative conclusion?

7) Assuming the Agency determined an ecological risk assessment with a greater degree of certainty concerning developmental effects of atrazine on amphibians were needed, please comment on EPA's conclusion that additional information is required to evaluate potential causal relationships between atrazine exposure and gonadal development. Please also comment on the added utility, if any, of additional information to interpret the shape of dose-response curves for potential developmental endpoints and the extent to which threshold or non-threshold response relationships can be quantified.

8) The Agency has developed a conceptual model from which to develop a set of study protocols for evaluating the potential effects of atrazine on gonadal development in amphibians. The Agency has proposed a research approach using focused, empirical, laboratory studies based on initial investigations with *X. laevis* followed by selective, confirmatory studies with frog species native to North America.

a) Please comment on the proposed sequence of study objectives.

b) Please comment on whether the Agency's first set of proposed studies has accounted for the major sources of uncertainty associated with the potential effects

of atrazine on anuran sexual differentiation. In addition to time to metamorphosis, gonadal abnormalities, and sex ratios in the proposed Phase I assays, please comment on any other endpoints that should be considered in this initial phase.

c) Please also comment on the range, spacing and number of atrazine concentrations that should be employed in the proposed testing sequence to resolve uncertainties in the shape and nature of dose-response relationships for any observed developmental effects.

d) Please comment on the Agency's recommendation that *X. laevis* be used as the primary biological model in the proposed studies and whether or not the mechanisms involved in sexual differentiation of the ranid and pipid species are sufficiently similar to predict effects and associated dose-response curves for *Rana* and/or to efficiently design *Rana* studies.

e) In this regard, are there important differences between the species to conclude that any affected developmental processes observed in *X. laevis* would not occur in *Rana*?

f) Alternatively, are there developmental pathways in *Rana*, but not in *X. laevis*, that raise concerns about using *X. laevis* as the primary biological model in any future atrazine studies?

g) Assuming *X. laevis* and *Rana* are sufficiently concordant from a toxicodynamic perspective with regard to potential developmental effects of atrazine, what critical toxicokinetic processes should be considered for extrapolating *X. laevis* dose-response relationships to *Rana* and/or for designing subsequent studies with *Rana*?

REFERENCES

Ankely, G. T., J. E. Tietge, D. L. DeFoe, K. M .Jensen, G. W. Holcombe, E. J. Durhan, S. A. Diamond. 1998. Effects of ultraviolet light and methoprene on survival and development of *Rana pipiens*. Environmental Toxicology and Chemistry 17: 2530-2542.

Birge, W. J., A. G. Westerman and J. A. Spromberg. 2000. Comparative toxicology and risk assessment of amphibians. Pages 727 - 791 *in* D. W. Sparling, G. Linder and C. A. Bishop, editiors. *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry Press.

Calabrese, E.. J. and L. A. Baldwin. 2001. Hormesis: a generalizable and unifying hypothesis. 2001. *Critical Reviews in Toxicology* 31 (4 & 5): 353 - 424.

Carr, J. A., A. Gentles, E. E. Smith, W. L. Goleman, L J. Urquidi, K. Thuett, R. J. Kendall, J. P. Giesy, T. S. Gross, K. R. Solomon, G. Van Der Kraak, 2003. Response of larval *Xenopus laevis* to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry* 22(2): 396 - 405.

40 CFR. 1997. Code of Federal Regulations 40. Parts 158 to 189 (Protection of the Environment). Office of the Federal Register National Archives and Records Administration.

Consent Decree (as amended) entered in Natural Resources Defense Council v. Whitman, Case Number C -99-3701 CAL, N. D. California (2002).

Crabtree, C.; E. E. Smith; J. A. Carr. 2003. Histology of the gonads and analysis of hormone levels in the native bull frog (*Rana catesbiena*) collected from agricultural areas in southern Iowa: pilot project. The Institute of Environmental and Human Health, Texas Technical University,

Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc. Laboratory Identification Number ECORISK Number TTU-02.

De Prado, R., N. Lopez-Martinez and J. Gonzalez-Gutierrez. 2000. Identification of two mechanisms of atrazine resistance in *Setaria faberi* and *Setari viridis* biotypes. *Pesticide Biochemistry and Physiology* 67(2): 114 - 124.

Duellman, W. E. and L. Trueb. 1994. *Biology of Amphibians*. Johns Hopkins University Press, Baltimore, MD.

DuPreez, L. H., A. M. Jooste and K. R. Solomon. 2003. Exposure of *Xenopus laevis* larvae to different concentrations of atrazine in semi-natural microcosms. School of Environmental Sciences and Development, Zoology Department, Potchefstroom University for CHE, Potchefstroom 2520, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01-D.

Giesy, J. P., M. Hecker, and P. D. Jones. 2003. South African analytical support – hormone and aromatase analysis (SA- 01C). Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-07*

Goleman, W. L. and J. A. Carr. 2003. Response of larval *Xenopus laevis* to atrazine exposure: assessment of metamorphosis and gonadal and laryngeal morphology. The Institute of

Environmental and Human Health, Texas Tech University, Texas Tech University Health Sciences Center, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number TTU-01.

Gray, J. A., N. E. Balke and D. E. Stoltenberg. 1996. Increased glutathione conjugation of atrazine confers resistance in a Wisconsin velvetleaf (*Abutilon theophrasti*) biotype. *Pesticide Biochemistry and Physiology* 55(3): 157 - 171.

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002*b*. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. *Environmental Health Perspectives* http://dx.doi.org/ *[Online 23 October 2002]*

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk. 2002*a*. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences* 99 (8): 5476 - 5480

Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002*c*. Feminization of male frogs in the wild: water-borne herbicide threatens amphibian populations in parts of the United States. *Nature* 419: 895 - 896. Abridged Version of Hayes *et al.* 2002b and not specifically reviewed in white paper.

Hayes, T. B. 1998. Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. *Journal of Experimental Zoology* 281: 373 - 399.

Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. A pilot study of response of larval *Rana clamitans* to atrazine exposure: assessment of metamorphosis and gonadal and laryngeal morphology and selected hormones and enzyme activities. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-03.

Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Response of *Xenopus laevis* to atrazine exposure: assessment of the mechanism of action of atrazine. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-04.

HED 2002. U. S. EPA Office of Pesticide Programs Human Health Effect Division Revised Human Health Risk Assessement for the Reregiatration Eligibility Decision (RED). April 16, 2002. OPP Docket #2002-0026. Docket Number 0012.

Huang, H, L Cai, BF Remo, and DD Brown. 2001. Timing of metamorphosis and the onset of the negative feedback loop between the thyroid gland and the pituitary is controlled by type II iodothyronine deiodinase in *Xenopus laevis*. *Proceeding of the National Academy of Sciences*, 98, 7348-7353

IRED Science Chapter for Atrazine: Environmental Fate and Effects Chapter, April 22, 2002 OPP Docket Number 2002-0026 Document Number 0005.

IRED. 2003. Interim Reregistration Eligibility Decision Science Chapter for Atrazine: Environmental Fate and Effects Chapter January 28, 2003. OPP Docket ID OPP-2003-0072; Document ID OPP-2003-0072-0010.

Jones, P. D., M. B. Murphy, M. Hecker, J. P. Giesy. 2003. Tissue pesticide residues and histology of the larynx and gonads in native green frogs (*Rana clamitans*) collected from agricultural areas in Michigan: hormone analysis. Aquatic Toxicology Laboratory, Michigan State University, 218C National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc. Laboratory Study ID: ECORISK Number MSU-02.

Miyashita, K, N Shimizu, S Osanai, and S Miyata. 2000. Sequence analysis and expression of the P450 aromatase and estrogen receptor genes in the *Xenopus* ovary. *J Ster Bioch Molec Biol*, 75, 101-107.

Nieuwkoop, P. D., and J. Faber. 1994. *Normal table of Xenopus laevis* (Daudin). North Holland Publishing Company, Amsterdam, Holland.

Petrini, S. F. Zaccanti. 1998. The effects of aromatase and 5-α reductase inhibitors, anitandrogens and sex steroids on Bidder's organs development and gonadal differentiation in *Bufo bufo* tadpoles. *Journal of Experimental Zoology* 280: 245 - 259.

Reeder, A. L., G. L. Foley, D. K. Nichols, L. G. Hansen, B. Wikoff, S. Faeh, J. Eisold, M. B. Wheeler, R. Warner, J. E. Murphy, and V. R. Beasley. 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). *Environmental Health Perspectives* 106 (5): 261 - 266

Reregistration Eligibility Decision Science Chapter for Atrazine: Environmental Fate and Effects Chapter, April 22, 2002 OPP Docket Number 2002-0026 Document Number 0005

Sanderson, J. T., J. Boerma, G. W. A. Lansbergen and M. van den Berg. 2002. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicology and Applied Pharmacology* 182: 44 - 54.

Sepulveda, M. S. and T. S. Gross. 2003. Characterization of atrazine exposures and potential effects in Florida ecosystems dominated by sugarcane agriculture: a reconnaissance survey of amphibians in south Florida for the assessment of potential atrazine effects. Department of Physiological Sciences, University of Florida, Caribbean Science Center, Gainesville, Florida. Sponsor: Syngenta Crop Protection, Inc. Study ID: ECORISK Number UFL-02.

Sive, H. L., R. M. Granger and R. M. Harland. 1998. *Early Development of Xenopus laevis, a Laboratory Manual*. Cold Springs Harbor Laboratory Press.

Smith, E. E., L. DuPreez, and K. Solomon. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: feasibility study for site characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, Texas (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01A*[4].

Smith, E., L DuPreez and K. Solomon. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: exposure characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental & Human Health, Texas Tech University, Lubbock, Texas 79490 (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Private Bag X6001, Potchefstroom 2520 (South Africa). Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01B*

Smith, E. E., L. DuPreez, and K. Solomon. 2003. Gonadal and laryngeal responses to field exposure of *Xenopus laevis* to atrazine in areas of corn production in South Africa. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01C*.

[4]*Individual DERs written on study, but report collectively reviewed as one of four component studies to the South African *Xenopus* investigations.

Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. Environmental Toxicology and Chemistry 21 (6): 1264 - 1267.

Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the testes. *Environmental Toxicology and Chemistry* 21 (3): 527 - 531.

Villalpando, I and H Merchant-Larios.1990. Determination of the sensitive stages for gonadal sex-reversal in *Xenopus laevis* tadpoles. *International Journal of Developmental Biology*, 34, 281-285.

Villeneuve, D. L., K. Coady, M. Hecker, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Methods development for the study of mechanism of action of atrazine in adult and metamophosing *Xenopus laevis* and *Rana clamitans*: aromatase induction. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-01.

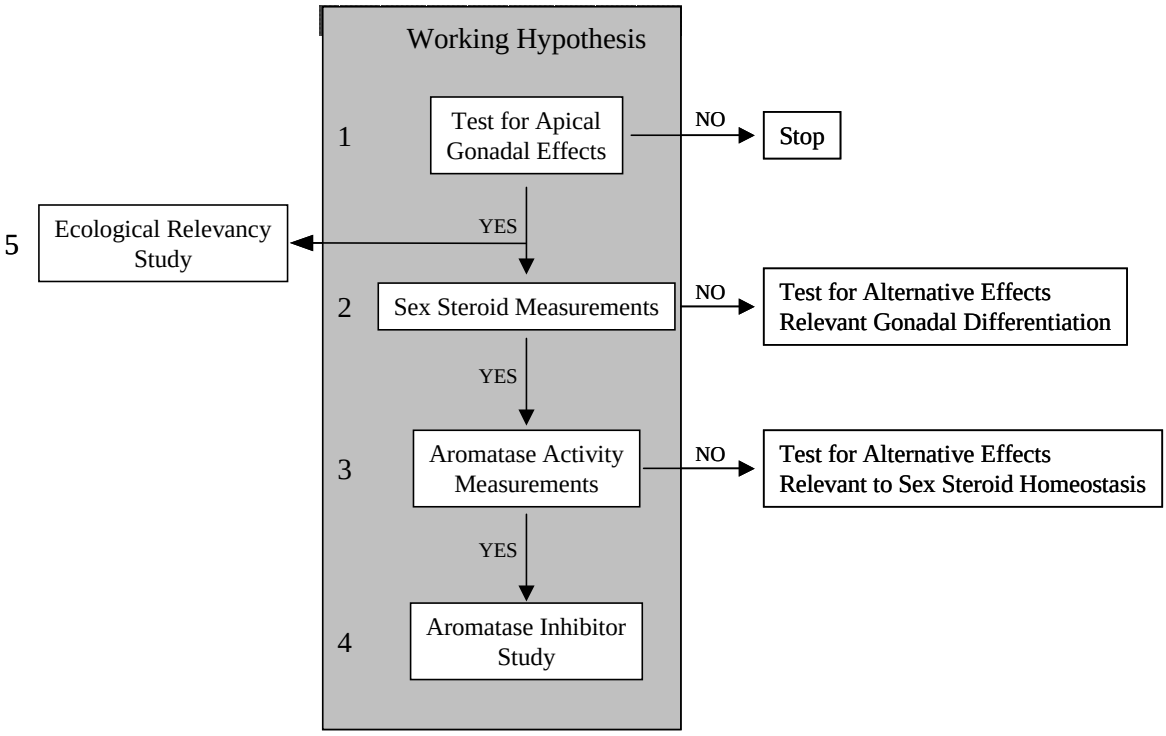

Figure 1. Scheme for laboratory studies to determine the effects of atrazine on gonadal differentiation in anuran amphibians. The grey block contains the working hypothesis for atrazine action on gonadal differentiation, which is partitioned into four logical phases. See text for details.

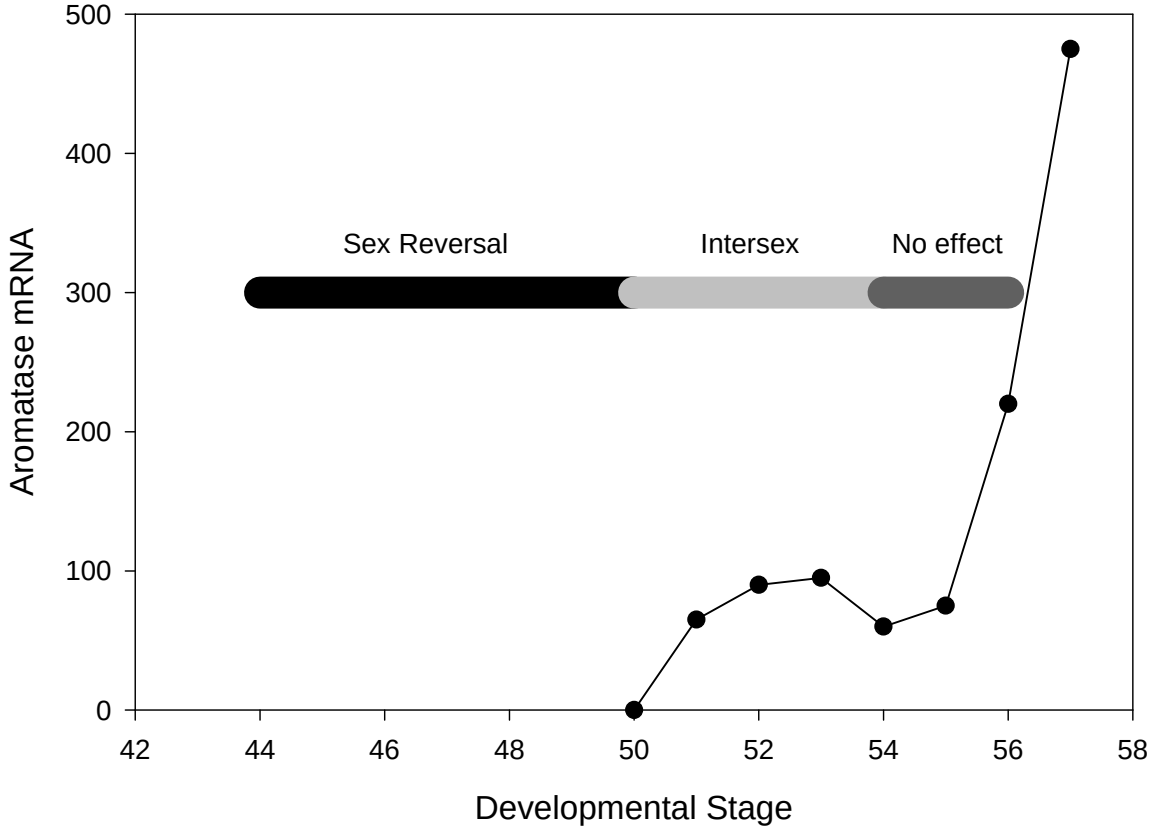

Figure 2. The effect of estradiol exposure on male gonads of different stages compared to the developmental expression of aromatase mRNA. The horizontal bar indicates that there are three periods of differential sensitivity to the feminizing effects of estrogen exposure for male *X. laevis* larvae (Villalpando and Merchant-Larios, 1990). The line indicates the relative expression of aromatase mRNA at different developmental stages (Miyashita *et al.* 2000).

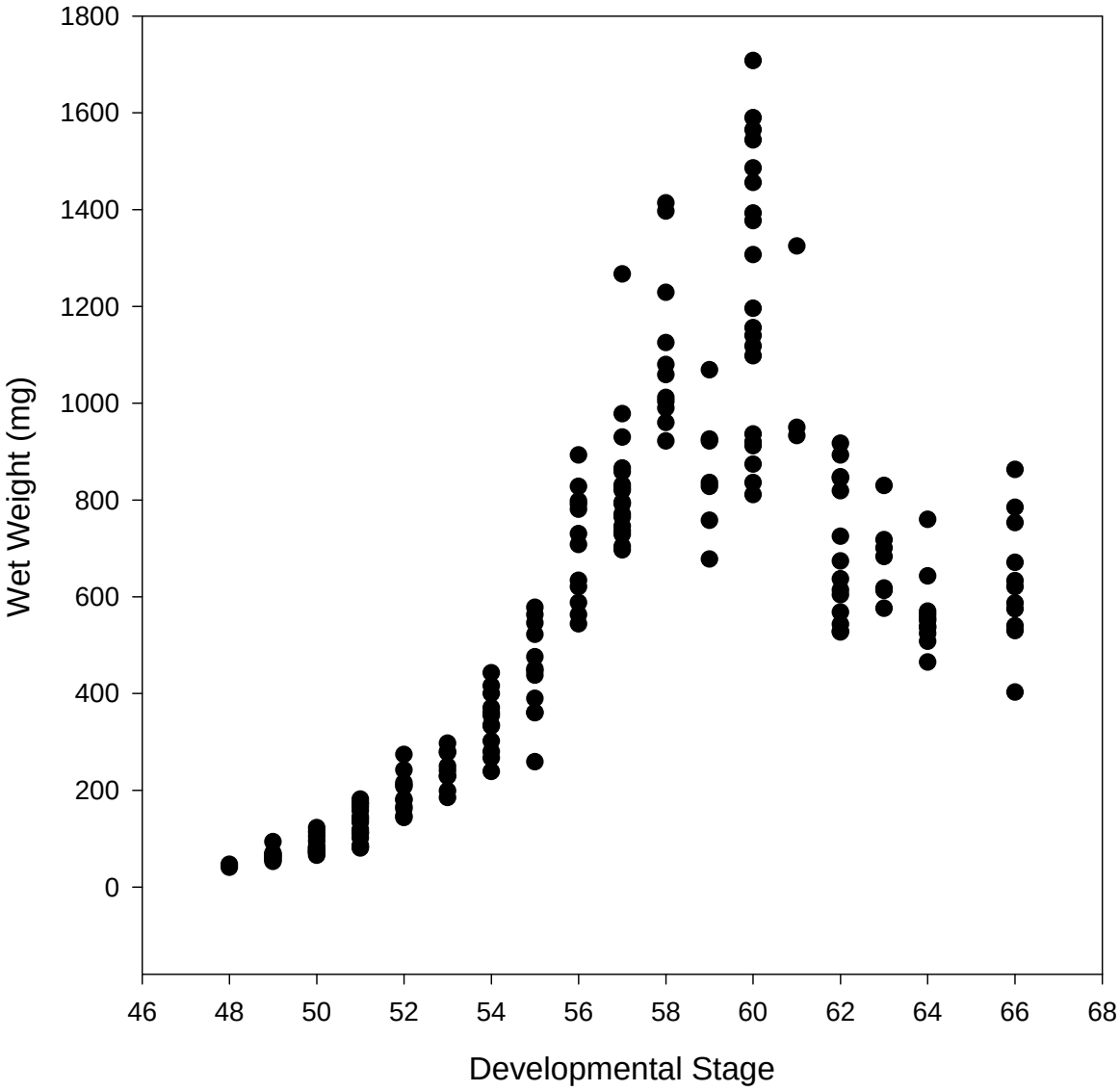

Figure 3. The relationship of weight to developmental stage of *X. laevis*. Each dot represents one organism (Tietge *et al.*, EPA Midcontinent Ecology Division, unpublished results.)